Thesis for the degree of Ph.D. at Glasgow University

by

Shelagh M. Foster.

SUMMARY

Part 1. (1) The production of metabolites during the growth of Bacterium coli follows a well defined pattern. The concentration of metabolites is very low during the early logarithmic growth phase and increases rapidly just before the onset of stationary phase. In non-proliferating cultures, subsequent to the cessation of growth, the metabolite concentrations decrease at a rate dependent on the degree of aeration.

(2) In cultures of different initial pH value, but with a similar degree of aeration, the metabolites produced, when expressed as a function of cell numbers in the culture, are the same in each culture regardless of pH.

(3) The continued utilization of glucose by stationary phase cultures depends on the initial pH of the medium and is independent of the degree of aeration during growth.

(4) The metabolism of glucose by cell suspensions of B. coli is affected by pH. This has previously been shown in terms of the end products produced from the glucose, (Tikka 1935,

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Stokes 1949). The rate of utilization depends on the pH : these are at a maximum at pH 7.1, and decrease as the pH is lowered.

(5) The relation of these findings to the dependence of glycolysis on the oxidation-reduction of diphosphopyridine nucleotide (DPN) is discussed.

Part 2. (1) The ability of washed cell suspensions to produce ethanol from glucose depends on the conditions of growth. A medium, containing traces of peptone and yeast extract, and incubated under anaerobic conditions, produces cells with the highest activity.

(2) The strain of B. coli used produced 2.5 μmoles acid fro 1 μmole glucose. The ethanol: acetic acid ratio was approxi- mately 1:1. The ethanol:glucose ratio was approximately 0.7

(3) The effect of inhibitors on the production of pyruvate and ethanol from glucose gives evidence that ethanol arises from pyruvate. No evidence of a second pathway via α-glycero phosphate could be obtained.

(4) Ethanol production is dependent on Coenzyme A(CoA). Thi was shown in experiments with a pantothenate requiring mutant of B. coli, and also by the treatment of cell-free extracts o

B. coli with Dowex 1.

(5) No ethanol was detected from the dissimilation of pyruva by cell suspensions of *B. coli*.

(6) Ethanol is utilized by cells of *B. coli*. The production of ethanol from glucose under the standard conditions adopted reached a maximum after 60 minutes and then declined. Also ethanol on incubation with a cell suspension of *B. coli* under an atmosphere of N2 is utilized. The fate of this ethanol is unknown. No relationship could be established between ethano and acetic acid concentrations.

(7) The alcohol dehydrogenase activity of cell-free extracts of *B. coli* was studied. These were partially purified by $(NH_4)_2\ SO_4$ fractionation and by elution from a calcium phosph gel.

(8) A cell-free extract of *B. coli* showed slight activity for the oxidation of acetaldehyde. This was dependent on CoA and

(9) The extracts could not reduce acetic acid or acetyl phosp *via* acetaldehyde to ethanol.

(10) These results supported the proposed pathway of ethanol production from glucose.

THESIS PRESENTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

THE UNIVERSITY OF GLASGOW

BY

SHELAGH MARGARET FOSTER,

DEPARTMENT OF BIOCHEMISTRY.

APRIL, 1956.

BIOCHEMICAL STUDIES WITH ESCHERICHIA COLI.

I PRODUCTS OF GLUCOSE METABOLISM DURING GROWTH.

II THE MECHANISM OF ETHANOL FORMATION.

SUMMARY.

Part 1. Products of glucose metabolism during growth.

(1) The production of metabolites during the growth of Escherichia coli follows a well defined pattern. The concentration of metabolites is very low during the early logarithmic growth phase and increases rapidly just before the onset of stationary phase. In non-proliferating cultures, subsequent to the cessation of growth, the metabolite concentrations decrease at a rate dependent on the degree of aeration.

(2) In cultures of different initial pH value, but with a similar degree of aeration, the metabolites produced, when expressed as a function of cell numbers in the culture, are the same in each culture regardless of pH.

(3) The continued utilization of glucose by stationary phase cultures depends on the initial pH of the medium and is independent of the degree of aeration during growth.

(4) The metabolism of glucose by cell suspensions of Esch. coli is affected by pH. This has previously been shown in terms of the end products produced from the glucose (Tikka 1935, and Stokes 1949). The rate of utilization and the amount of glucose utilized depends on the pH: these are at a maximum at pH 7.1, and decrease as the pH is lowered.

(5) The relation of these findings to the dependence of glycolysis on the oxidation-reduction of diphosphopyridine nucleotide (DPN) is discussed.

Part 2. The mechanism of ethanol formation.

(1) The ability of washed cell suspensions to produce ethanol from glucose depends on the conditions of growth. A medium containing traces of peptone and yeast extract, and incubated under anaerobic conditions, produces cells with the highest activity.

(2) The strain of Esch. coli used produced 2.5 µmoles acid from 1 µmole glucose. The ethanol:acetic acid ratio was approximately 1:1. The ethanol:glucose ratio was approximately 0.7:1.

(3) The effect of inhibitors on the production of pyruvate and ethanol from glucose gives evidence that ethanol arises from pyruvate. No evidence of a second pathway via α-glycerophosphate could be obtained.

(4) Ethanol production is dependent on Coenzyme A (CoA). This was shown in experiments with a pantothenate requiring mutant of Esch. coli, and also by the treatment of cell-free extracts of Esch. coli with Dowex 1.

(5) No ethanol was detected from the dissimilation of pyruvate by cell suspensions of Esch. coli.

(6) Ethanol is utilized by cells of Esch. coli. The production of ethanol from glucose under the standard conditions adopted reached a maximum after 60 minutes and then declined. Also ethanol on incubation with a cell suspension of Esch. coli under an atmosphere of N_2 is utilized. The fate of this ethanol is unknown. No relationship could be established between ethanol and acetic acid concentrations.

(7) The alcohol dehydrogenase activity of cell-free extracts of Esch. coli was studied. These were partially purified by $(NH_4)_2SO_4$ fractionation and by elution from a calcium phosphate gel.

(8) A cell-free extract of Esch. coli showed slight activity for the oxidation of acetaldehyde. This was dependent on CoA and DPN.

(9) The extracts could not reduce acetic acid or acetyl phosphate via acetaldehyde to ethanol.

(10) These results supported the proposed pathway of ethanol production from glucose.

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PART I.

INTRODUCTION.

I N T R O D U C T I O N.

There exists a considerable literature in which the products of fermentation are claimed to have been investigated during bacterial growth. Many such papers are listed by Stephenson (1949), who states that the work of Scheffer (1928) provides the most complete and reliable quantitative data on fermentations in growing cultures. Examination of the methods used by these workers reveals that the growth of the cultures was not followed quantitatively and that the determinations of the fermentation products were carried out after several days of 'growth' usually in complex media. Scheffer (1928) carried out his analysis after Escherichia coli had 'grown' for five days in a peptone-glucose medium. Such media would ensure reasonably rapid growth and attainment of the stationary phase in minimum periods of time. Under these circumstances it seems fairly certain that the products analysed after several days would be the combined result of metabolic activities by both growing and non-proliferating cells subsequent to the cessation of growth and, therefore, not necessarily related to the processes of active cell division. Accordingly, little information is available concerning the metabolic products that appear in culture media during cell proliferation.

The only published results which refer unambiguously to active cell division are those of Hagley, Dawes and Morrison (1950a, 1951) who investigated the production of pyruvic acid during the growth of Aerobacter aerogenes in defined ammonium salt media containing glucose and various organic acids as the sole source of carbon. The present work extends these observations to the production of other metabolites from glucose including lactate, acetate, formate, α-ketoglutarate, ethanol and also of pyruvate during the growth of Esch. coli cultures. The initial pH of the medium, and the degree of aeration during growth, affect the stationary population of cultures. The effect of variations in these two factors on the production of metabolites during growth has been studied in an attempt to investigate their site of action in relation to the energy-yielding reactions of the cell. Glucose metabolism and some aspects of bacterial growth will be reviewed prior to presenting the results.

GLUCOSE METABOLISM.

The Glycolytic Pathway.

Fermentation in yeast and muscle occurs *via* a series of reactions given in the Embden-Meyerhof-Parnas scheme. The primary mechanism of this process, which results in the production of pyruvic acid, is common to both fermentations, although their subsequent metabolism of pyruvate differs. Comparative biochemistry has shown that many basic patterns exist in the metabolism of all forms of life, and the fact that the primary pathway of glycolysis was common to both yeast and muscle, led to the expectation that this same pathway might well function in bacteria.

Aubel (1926) demonstrated that pyruvate was produced by *Escherichia coli* grown on glucose. Cook (1930) added the ketone fixative, sodium bisulphite, to the fermentation of glucose by washed cell suspensions of *Esch. coli* and found that pyruvic acid accumulated under these conditions. Wood and Werkman (1934), Wood, Stone and Werkman (1937) and Davies and Stephenson (1941) have shown that *Propionibacterium arabinosum* and *Clostridium acetobutylicum* produce pyruvate from glucose. Werkman, Zoellner, Gilman and Reynolds (1936) first reported the isolation and identification of phosphoglyceric acid as an intermediate in the anaerobic dissimilation of glucose

by Citrobacter freundii, and then Stone and Werkman (1936(a), 1936(b), 1937) isolated phosphoglyceric acid from glucose dissimilations by Esch. coli, Aerobacter indologenes, Propionibacterium shermani, P. arabinosum and Propionibacterium pentosaceum. In all these experiments sodium fluoride was used as an inhibitor. Tikka (1935) showed that phosphoglyceric acid was broken down by Esch. coli to normal end products of glucose fermentation. He also showed that washed cell suspensions of Esch. coli converted both glucose and hexose diphosphate to the same end products and both fermentations were influenced in an identical way by the pH of the medium. Endo (1938) used an acetone dried powder of Esch. coli and obtained pyruvic acid and inorganic phosphate from glucose and hexose diphosphate, and in the presence of sodium fluoride obtained phosphoglyceric acid.

Still (1940(a)) made cell-free preparations from Esch. coli which contained triosephosphate dehydrogenase (6). The aldolase (5)* of Esch. coli was studied in detail by Utter and Werkman (1941). These same workers (1942(a) and (b)) studied the conversion of 3-phosphoglyceric acid to 2-phosphopyruvic acid, and the transfer of phosphate

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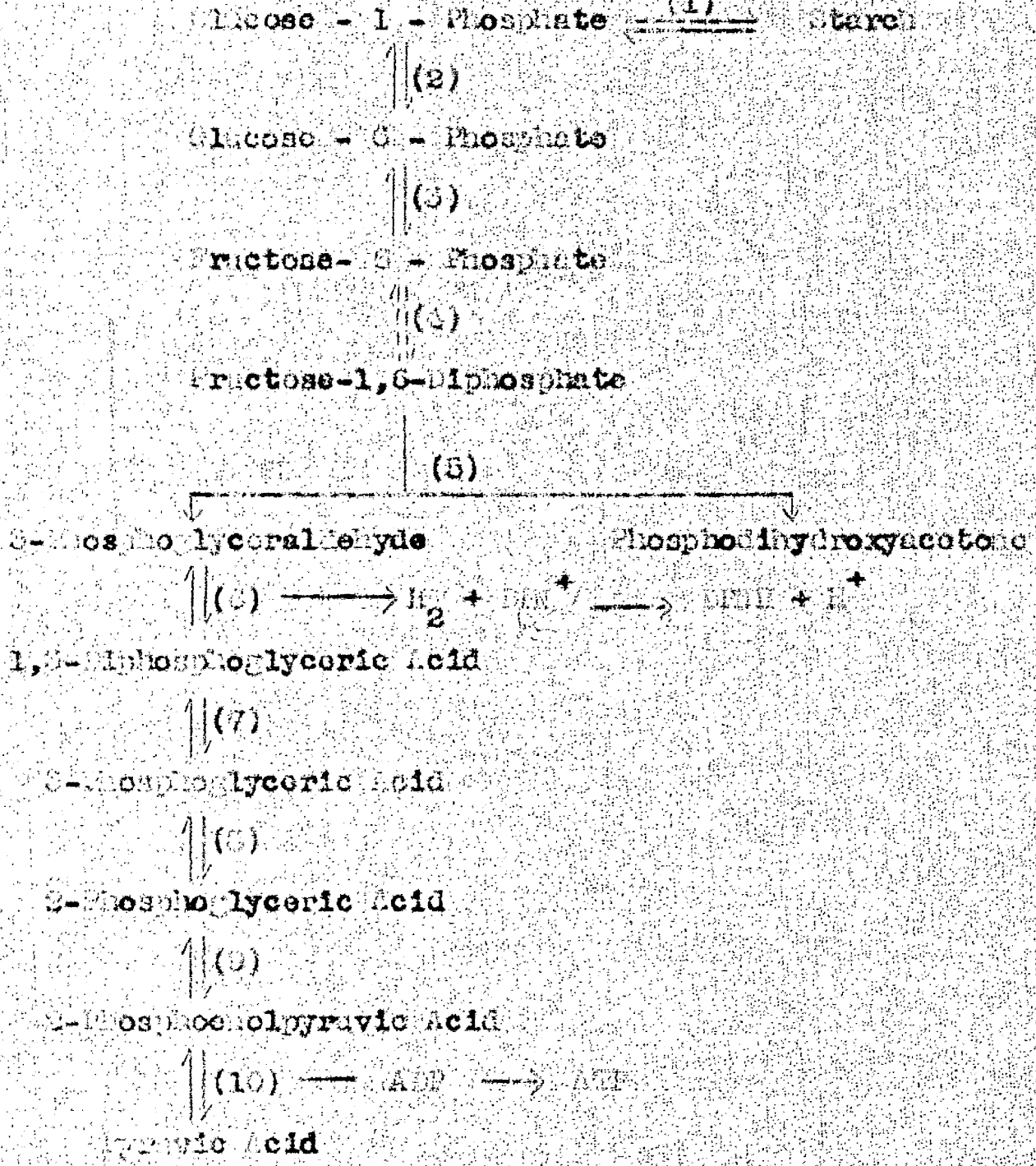
* Ringed figures refer to the correspondingly numbered reactions illustrated in Chart 1, facing p.3.

The Glycolytic Pathway of Glucose Metabolism.

The Reactions of the Embden-Meyerhof-Parnas Scheme found in *Escherichia coli*.



from the latter to adenylic acid. Thus the existence of phosphoglyceromutase (8) and enolase (9) was established. Doudoroff, Hassid, Putman and Potter (1949) showed that dried preparations of Esch. coli contained the enzymes, phosphorylase (1), phosphoglucomutase (2) and phosphohexose isomerase (3). The addition of glucose-1-phosphate to the extract resulted in an equilibrium mixture of glucose-1-phosphate, glucose-6-phosphate and fructose -6-phosphate. The enzymes of the primary mechanism which have been found in Esch. coli are shown in the scheme opposite.

The Pasteur Effect.

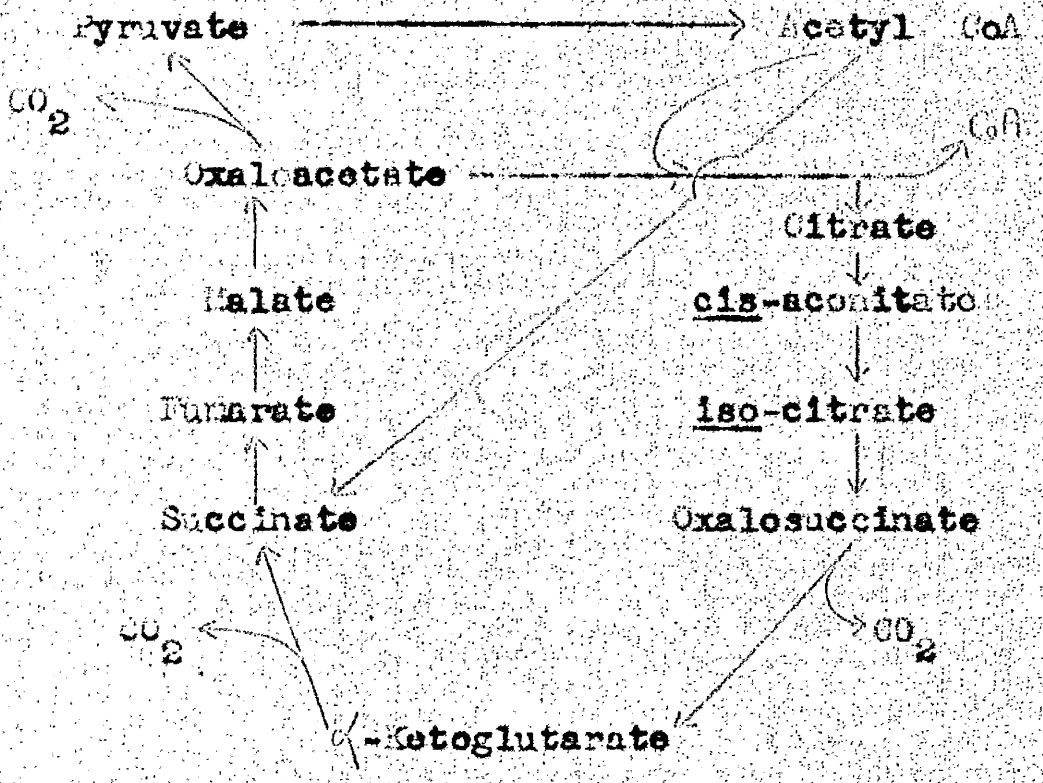
Pasteur (1861) compared the growth of yeast in two flasks, one with access to the air, and the other anaerobically sealed. He found that one 'part' of yeast grown in the presence of air required 4 to 10 'parts' of sugar, while the same amount of yeast under anaerobic conditions required as much as 60 to 80 'parts' of glucose. Thus he concluded that yeast required oxygen for life, and under conditions where it is denied free oxygen it must obtain it by the breakdown of fermentable material. This inhibition of fermentation by oxygen was called the Pasteur Effect. Pasteur failed to distinguish between the requirements of the cell for oxygen and for energy. In the

presence of oxygen the carbohydrate source can be completely oxidized with a full yield of energy, whereas in the absence of oxygen the intermediates of the breakdown must act as hydrogen acceptors and therefore accumulate, resulting in a wasteful and incomplete breakdown of the sugar. The energy relations of the change from fermentation to oxidation have been discussed by Lipmann (1942).

The question now arises, how does oxygen inhibit the glycolytic pathway, or how is glucose metabolized in the presence of oxygen. There are three suggested pathways for glucose oxidation, and these will be considered in turn.

Firstly, there is the glycolytic conversion of glucose to pyruvate, followed by its oxidation <u>via</u> the tricarboxylic or dicarboxylic acid cycles. Aubel (1926) found that pyruvate was produced from glucose under both oxidative and fermentative conditions. From the Embden Meyerhof glycolysis scheme it is seen that the oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid is linked with the reduction of diphosphopyridine nucleotide (DPN). This coenzyme must be reoxidized for glycolysis to continue. In the absence of oxygen as a hydrogen acceptor, some organic hydrogen acceptor, for example acetaldehyde or pyruvic acid, must reoxidize the reduced DPN. In the presence of oxygen, which will act as the

The Dicarboxylic and Tricarboxylic Acid Cycles.



hydrogen acceptor, the DPN will be regenerated without the reduction of pyruvate or any of its products, (Meyerhof (1942)). This will consequently prevent the formation of reduced fermentation end-products.

The Di- and Tri-Carboxylic Acid Cycles.

Pyruvic acid formed by glycolysis is then free to be oxidized via the dicarboxylic acid or tricarboxylic acid cycles, which are shown opposite. Novelli and Lipmann (1950) prepared a cell-free extract from Esch. coli which synthesized citrate from acetate, oxaloacetate, adenosine triphosphate (ATP) and Coenzyme A (CoA). On the other hand, Ajl and Kamen (1951) studied the simultaneous oxidation of labelled acetate by Esch. coli with unlabelled succinate, pyruvate, α-ketoglutarate, fumarate, malate and oxaloacetate singly or in combination and found that there was incorporation of labelled carbon into all substrates except α-ketoglutarate. Ajl, Kamen, Hanson and Wong (1951) repeated these experiments with Micrococcus lysodeikticus and found that the labelled carbon from acetate was trapped to the same extent in all the carriers, including α-ketoglutarate, showing that in this case α-ketoglutarate and the C_4 dicarboxylic acids participated equally in the oxidation of acetate. Ajl and Wong (1951) repeated this work with Aerobacter aerogenes

and found evidence once again in favour of the tricarboxylic acid cycle. They also found, however, that when unlabelled acetate was incubated with labelled dicarboxylic acids, labelled acetate was found at the end of the experiment, which is difficult to explain in terms of the tricarboxylic acid cycle, but is expected for the dicarboxylic acid one. Simultaneous adaptation experiments, based on those of Stanier (1947), showed that citrate adapted cells oxidized acetate, but acetate adapted cells would not oxidize citrate. Thus on the basis of these results, it would appear that citrate is not involved in acetate oxidation.

Lagley, Lawes and Morrison (1951) studied the kinetics of pyruvate production by A. aerogenes. For aerated washed suspensions of cells trained to metabolize glucose, malate, fumarate, or succinate respectively, the rates of pyruvate production from various substrates were in the order: rate in malate > fumarate > succinate > acetate. Only cells which were trained to metabolize acetate produced pyruvate from acetate as fast as from succinate and fumarate. Such cells produced pyruvate immediately they were aerated in the presence of a suitable substrate. An accumulation of pyruvate in the early stages required either the production of oxaloacetate in

excess of the requirements of the tricarboxylic acid cycle, or the presence of appreciable amounts of oxaloacetate initially. The demands of the cycle that one mole of oxaloacetate must be consumed for each mole produced, ruled out the former suggestion, and since no pyruvate was produced when cells were aerated in the absence of substrate, there could have been no initial concentration of oxaloacetate. These results were not compatible with the existence of the tricarboxylic acid cycle in bacteria, but Bagley, Morrison and Dawes (1951a,b) pointed out that they could be explained if the dicarboxylic acid cycle was accepted. They suggested that this cycle might function, not only to provide energy for growth, but also to provide the pyruvate necessary for growth. Krebs, Gurin and Eggleston (1952) studied acetate oxidation in baker's yeast treated with dry ice, and concluded that although all the component reactions of the tricarboxylic acid cycle were present in yeast, the cycle did not constitute the main pathway for acetate oxidation. This was based on the results of simultaneous oxidation experiments with labelled acetate in the presence of non-labelled second substrates from the cycle, and also on the fact that although succinate oxidation was competitively inhibited by malonate, the oxidation of acetate was

unaffected under identical conditions. Those authors concluded that the component reactions of the cycle probably supply intermediates for organic synthesis, rather than energy. Experiments carried out by Citinelli, Ehrensvard, Reio, Saluste and Stjernholm (1951) supported the assumption that the synthesis of the carbon skeleton of amino acids, for example glutamic acid, aspartic acid and alanine, involves the reactions of the tricarboxylic acid cycle. McQuillen (1954) reported findings which indicated that the tricarboxylic acid cycle is of fundamental importance in furnishin intermediates for amino acid synthesis in Esch. coli but plays only a minor part in carbohydrate oxidation. In contrast to this, Silvarg and Davis (1954) worked with mutants of Esch. coli and reported that in their opinion the tricarboxylic acid cycle not only functioned in Esch. coli for biosynthetic purposes but was the only substantial means for combusting acetate.

Saz and Krampitz (1954) studied the oxidation of labelled acetate in the presence of monisotopic α-keto-glutarate and succinate, and their results were in direct accord with the mechanism of the tricarboxylic acid cycle. They pointed out that Ajl et al. (1951) had already reached this same conclusion using the same organism, Micrococcus lysodeikticus, but that this previous conclusion was open to question. It had been based on results obtained

using carriers which had failed to achieve a reasonable degree of equilibration in the system. Swim and Krampitz (1954a) failed to demonstrate any incorporation into an α-ketoglutarate carrier of the isotope from labelled acetate oxidized by Esch. coli. When the acetate was oxidized in the absence of added carriers, citrate, α-ketoglutarate, succinate, fumarate and malate were isolated and found to be in isotopic equilibrium with each other, the respiratory CO_2 and the residual acetate. This was taken as evidence in favour of the tricarboxylic acid cycle. Degradation of the succinate formed from labelled acetate by Esch. coli in further experiments by Swim and Krampitz (1954b) showed that this was formed via the tricarboxylic acid cycle, and not by a condensation of two acetate molecules.

Stone and Wilson (1952) prepared cell-free extracts of Azotobacter vinelandii which oxidized acetate, pyruvate and the acids of the tricarboxylic acid cycle without any demonstrable lag period, whereas the parent cell suspensions either had long lag periods or were completely inactive on these substrates. They found a clear cut 'sparking' effect of oxaloacetate on the oxidation of acetate and pyruvate by these extracts. These oxidations were also stimulated by the addition of CoA. They concluded that the primary pathway of acetate oxidation by

A. vinelandii is the tricarboxylic acid cycle.

Individual enzymes of the tricarboxylic acid cycle have been studied. Kornberg and Ajl (1952) isolated a triphosphopyridine nucleotide (TPN) specific iso-citric dehydrogenase from Escherichia freundii: α-ketoglutarate was produced from iso-citrate. Wheat and Ajl (1954) obtained crude extracts of Esch. coli which contained aconitase and iso-citric dehydrogenase, which converted citrate to α-ketoglutarate. In favour of the dicarboxylic acid cycle we have the enzyme which synthesized succinate from acetate, and catalyzed the reverse reaction, studied by Slade and Werkman (1943).

It would appear from the evidence now available that bacteria utilize both pathways in the oxidation of acetate. Barron and Ghiretti (1953) have put forward a possible explanation why organisms should appear to possess both cycles. They suggest that all cells oxidize acetate via the tricarboxylic acid cycle, but also possess the dicarboxylic acid cycle to provide the oxaloacetate required for citric acid formation. Umbreit (1953) has postulated that there are even more respiratory cycles than are at present known, and postulates the presence of a cycle which depends on the condensation of C_3 and C_4 compounds to give a C_7 compound.

The Hexose Monophosphate Shunt.

Secondly we have the stepwise oxidation of glucose-6-phosphate through the hexose monophosphate pathway. While purifying yeast aldolase, Warburg (1948) found that it was inhibited by cysteine and other inhibitors which combine with heavy metals, and he concluded that it was a ferroprotein. The oxidation of the prosthetic group (Fe^{++} to Fe^{+++}) would inhibit the enzyme and this was offered as a possible explanation of the Pasteur Effect. In the presence of oxygen hexose diphosphate would accumulate, and the oxidation of glucose would then proceed via the oxidation of a hexose monophosphate, either fructose or glucose phosphate. Engelhart and Sekov (1948), on the other hand, suggested that the Pasteur Effect was due to the phosphohexokinase enzyme, which was inhibited by the addition of cytochromes. If this is the case, fructose monophosphate would accumulate and the oxidation would proceed via glucose monophosphate. Warburg, Christian and Griese (1935) showed that yeast enzymes oxidized glucose-6-phosphate to 6-phosphogluconate (13)*. Baeron and Friedemann (1941) found that bacteria which were unable to ferment glucose oxidized it, and that glucose

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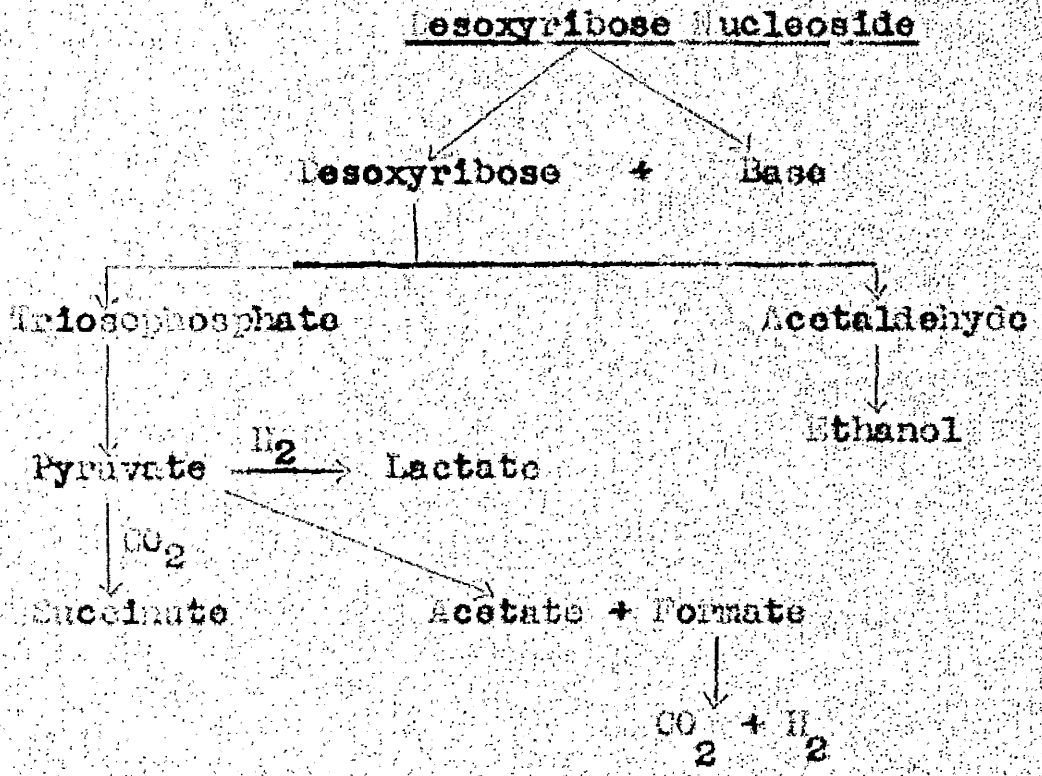
*This and subsequent ringed figures refer to the correspondingly numbered reactions in Chart facing page 19.

monophosphate was an intermediate. Dickens (1938a) obtained a yeast extract which fermented ribose-5-phosphate, at the same rate as glucose and glucose monophosphate, into 1 mole alcohol, 1 mole CO_2, 1 mole free phosphoric acid and an unidentified product. He went on to prepare (1938b) another yeast extract which oxidized phosphohexonic acid to a mixture of phosphoketohexonate and phosphopentonic acid.

Fred, Peterson and Anderson (1921) studied the fermentation of arabinose and xylose by the lactic acid bacteria, and concluded that the main line of fermentation was a simple cleavage into acetic and lactic acid (7). Stanier and Adams (1944) studied the fermentation of glucose and xylose by Aeromonas hydrophilia and found that they both yielded, 2,3-butylene glycol, ethanol, acetic acid, lactic acid, CO_2 and H_2. This was regarded as evidence against a C_2 - C_3 cleavage of the xylose, because it necessitated the formation of CO_2 from a C_2 compound. Hacker (1948), however, obtained an enzyme in bacterial extracts which yielded triosephosphate from ribose-5-phosphate, and another which synthesized a pentose phosphate from glycoaldehyde and triosephosphate (7). Kenshal, Jowett and Walker (1951) isolated glycoaldehyde from cultures of Acetobacter aceticemum grown on D-xylose

The Degradation of Desoxyribose Nucleosides.

Hoffman and Lampen (1952).



and L-arabinose, while Harman and Schlenk (1951) found that both glycolaldehyde and its phosphate ester combined with triosephosphate to give pentose phosphate (7). Racker (1951) prepared extracts from Esch. coli which catalyzed the reversible reaction

Glyceraldehyde Phosphate + Acetaldehyde $\rightleftharpoons$ Desoxypentose Phosphate

and suggested that the metabolism of pentoses and desoxypentoses may be linked through the triose phosphates and that the nucleic acids may yield energy through this mechanism (10)(11). Hoffman and Lampen (1952) from the results of the degradation of thymine desoxyribose, proposed the scheme opposite as a probable pathway for the degradation of desoxyribose nucleosides (12). Lampen, Gest and Bowden (1950) and Gest and Lampen (1951) allowed Lactobacillus pentosus to degrade 1-^{14}C-xylose with the following results:

1-^{14}C-Xyloce $\longrightarrow$ 1-^{14}C-Acetate + Lactate

x_{C}-C-C-C-C x_{C}-C C-C-C

1 2 3 4 5 1 2 3 4 5

This supported the split of pentoses into a C_2 and C_3 compound, and they suggested that a 2-ketopentose was formed as an intermediate. Kappoport, Barker and Hassid (1951) confirmed these findings. They found that when 1-^{14}C-L-arabinose was fermented by Lactobacillus pentoaceticus

the C_1 and C_2 carbons gave acetic acid, labelled in the methyl group, and that lactic acid resulting from the remaining three carbon atoms was unlabelled. Sable (1951) found that ribose-5-phosphate reduced TPN in the presence of a yeast extract fraction only after a considerable lag period. If, however, the sugar and the yeast extract were incubated before the addition of TPN, then the lag was abolished. Thus they concluded that some other compound, and not the ribose-5-phosphate, was responsible for the reduction of TPN.

Hutting and Carson (1952 a and b) found that when Esch. coli fermented xylose at a pH below 5.8, 1 mole of glucose yielded more than the one mole of lactate expected from a C_2 - C_3 split, and they concluded, and confirmed by tracer studies, that lactate arose from a C_2 moiety by condensation with a C_1 compound (15).

Racker (1948) found a TPN dependent enzyme in bacterial extracts which oxidized phosphogluconate to pentose phosphate (5). Horecker and Smyrniotis (1950) found that 6-phosphogluconate on oxidation gave ribose-5-phosphate and a second pentose phosphate (5) and (6). This second compound was found to be a precursor of ribose-5-phosphate and they proposed the scheme:

6-Phosphogluconate $\xrightarrow{TPN}$ (3-keto-6-phosphogluconate) $\longrightarrow$ ribulose-5-phosphate $\longrightarrow$ ribose-5-phosphate.

Horecker and Smyrniotis (1951) isolated from yeast, a phosphogluconic acid dehydrogenase, which quantitatively converted 6-phosphogluconate to pentose phosphate and CO_2 in the presence of TPN (5). Horecker, Smyrniotis and Seegmiller (1951) subjected this pentose phosphate to chromatography and chemical analysis, and found that it consisted of an equilibrium mixture of ribose-5-phosphate (70-80%) and ribulose-5-phosphate. The ribose-5-phosphate was formed from ribulose-5-phosphate by the action of pentose isomerase which was present in the yeast extract (6). A system oxidizing 6-phosphogluconate in the presence of TPN was demonstrated in Esch. coli extracts by Scott and Cohen (1951)(5). Ribose-5-phosphate, arabinose-5-phosphate and an unknown phosphate ester were found as products of the reaction.

Roberts and Wolffe (1951) found that when unlabelled fructose-6-phosphate, glucose-1-phosphate, and glucose-6-phosphate were incubated with labelled inorganic phosphate and Esch. coli, no activity could be detected in the oxidation products. Therefore, they concluded that fructose-1,6-diphosphate was not an intermediate. This was confirmed using labelled fructose-6-phosphate and fructose-

1,6-diphosphate. Labelled fructose-6-phosphate gave high activity in pentose phosphate, and in nucleic acids (9) and (10). Labelled fructose-1,6-diphosphate was not utilized. Two thirds of the phosphorous from α-glycerophosphate appeared in the nucleic acids. They also stated that this fructose-6-phosphate was not metabolized via glucose-6-phosphate.

Cohen (1951) isolated gluconokinase from cells of Esch. coli which had been adapted to the oxidation of gluconate (4). This catalyzed the reaction:

Gluconate + ATP $\xrightarrow{Mg}$ 6-Phosphogluconate + ADP.

Non-adapted cells were found to contain the enzymes for the oxidation of glucose and of 6-phosphogluconate, which gives ribose-5-phosphate and triosephosphate.

Seegmiller and Horecker (1952) identified as one of the products of pentose phosphate oxidation, glucose-6-phosphate (6)(5)(13). Lampen (1953) has discussed the possibility that pentoses might be metabolized by initial condensation with a C_1 compound which is then split into two triosephosphates. Horecker (1953) has provided experimental evidence that ribulose phosphate, under the action of phosphogluconic acid dehydrogenase from yeast, with TPN and bicarbonate yielded phosphogluconate (6). Gibbs and Horecker (1954a and b) have studied the mechanism of pentose phosphate conversion to hexose phosphate with a

pea leaf and pea root preparation.

Horecker and Smyrniotis (1952) purified an enzyme from rat liver which converted pentose phosphate to sedoheptulose phosphate (7)(14). They suggested that there was a combination of $C_2 + C_2 \longrightarrow C_4$, which combined with C_3 to form the C_7 sugar. Horecker and Smyrniotis (1954) isolated yeast transaldolase which catalyzed the reaction (16):

Sedoheptulose 7-phosphate + Glyceraldehyde-3-phosphate

$\rightleftharpoons$ Fructose-6-phosphate + Tetrose phosphate

This tetrose phosphate was shown to be D-erythrose-4-phosphate. Wood and Schwert (1954) detected sedoheptulose phosphate formation from ribose-5-phosphate and ribulose-5-phosphate by Pseudomonas fluorescens (6)(7) and (14).

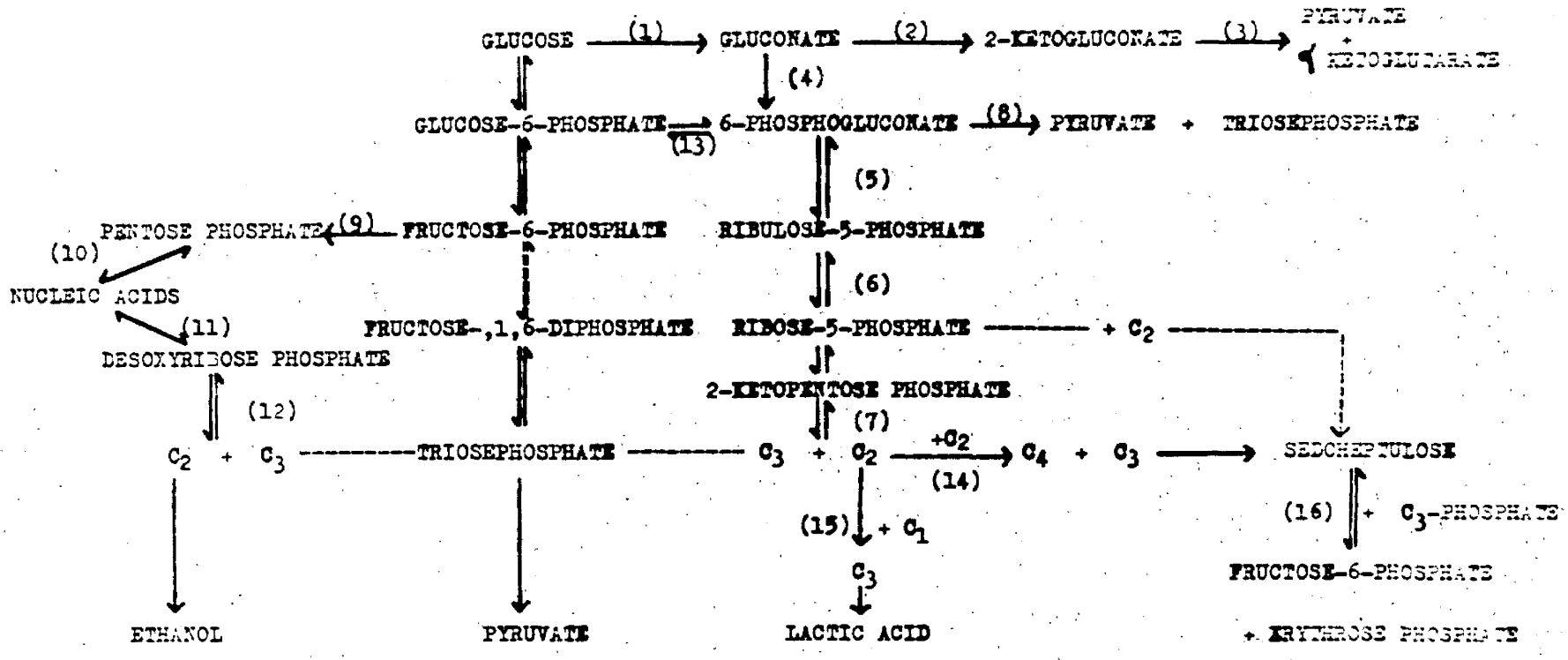
Gluconate Oxidation.

The third suggested pathway for glucose oxidation is the stepwise oxidation of non-phosphorylated compounds involving gluconate and 2-ketogluconate as intermediates. The finding of Barron and Friedemann (1941) that glucose oxidation in Pseudomonas aeruginosa was not inhibited by fluoride first indicated the existence of an oxidative pathway other than one involving the glycolytic system. Lockwood, Tabenkin and Ward (1941), studying the glucose

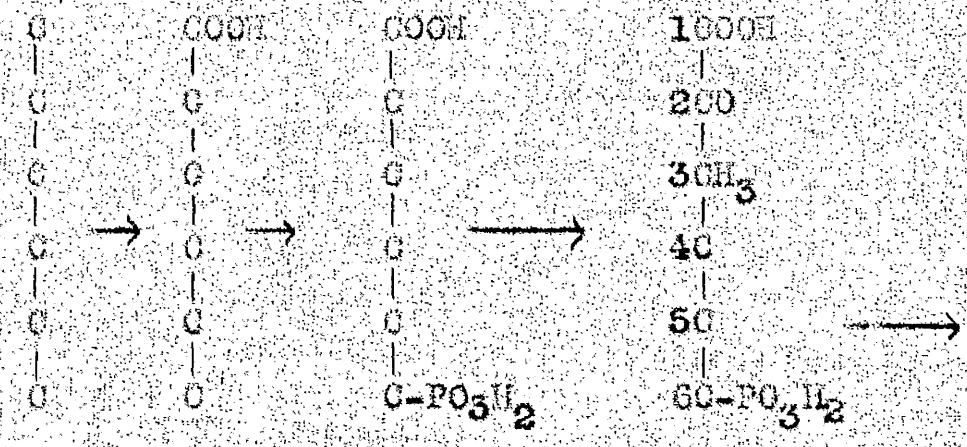
metabolism of Pseudomonas and Phytomonas species in aerated cultures, found that gluconic acid and 2-ketogluconic acid were formed from glucose (1)(2). The pathway for the oxidation of 2-ketogluconic acid has not been clarified. Koepsell (1950) described conditions under which he obtained pyruvate and α-ketoglutarate from cultures of Ps. fluorescens grown in the presence of 2-ketogluconate, but whether these lie on the normal pathway has so far not been established (3). Stokes and Campbell (1951) excluded the possibility of phosphorylated intermediates in the oxidation of glucose to 2-ketogluconic acid. Studying the oxidation of glucose and gluconic acid by dried cells of Ps. aeruginosa, they showed that 1 μ mole glucose required 1 μ mole oxygen, whereas 1 μ mole gluconic acid required 0.5 μ mole oxygen. The 2-ketogluconate was not oxidized. They could not demonstrate any dependence on ATP, nor any inhibition by sodium fluoride or iodoacetate, nor detect any phosphorylated compounds.

Entner and Stanier (1951) further established the oxidation of glucose and gluconic acid to 2-ketogluconic acid in Ps. fluorescens, but gave evidence to show that the oxidation of these compounds cannot proceed chiefly via this compound. Entner and Pondoroff (1952) have observed the formation of carboxyl-labelled pyruvate from 1-^{14}C glucose and 1-^{14}C gluconic acid by Pseudonomas

THE PATHWAYS OF GLUCOSE METABOLISM



saccharophila. Cell-free extracts of this organism form pyruvate and triosephosphate from 6-phosphogluconate (6). They therefore suggest the following scheme:



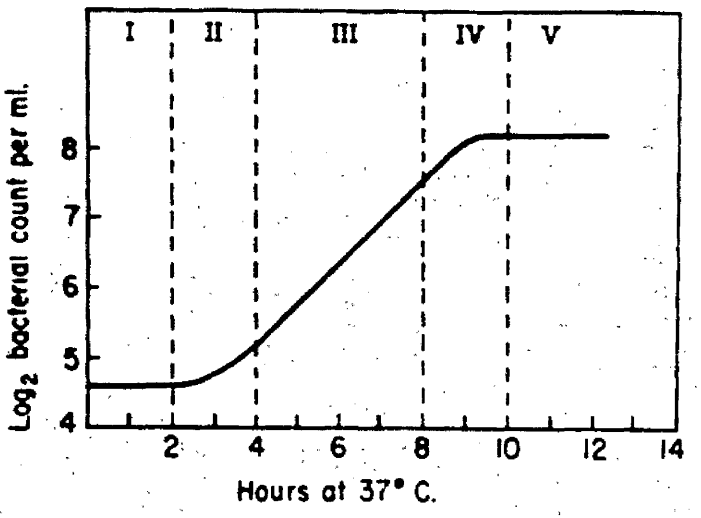
This split is definitely distinct from that of the Embden Meyerhof pathway where the pyruvate would be numbered

$CH_3.CO.COOH$ and $CH_3.CO.COOH$

1 2 3 4 5 6

Further evidence that gluconic acid is metabolized through 6-phosphogluconate was provided by Cohen (1951) who isolated the gluconokinase enzyme from Esch. coli which was adapted to gluconic acid oxidation.

The pathways of glucose metabolism are summarized opposite.



Growth curve, conventionalized. *Escherichia coli*, 37° C., nutrient broth (adapted from Monod).

BACTERIAL GROWTH.

If a number of living bacteria are inoculated into a suitable nutrient medium, their subsequent growth follows a well defined cycle. A conventionalized growth curve, for Esch. coli grown in nutrient broth at 37°C, which has been adapted from Monod (1949) is shown opposite. In it five phases of growth can be distinguished:

(1) lag phase

(2) acceleration phase

(3) logarithmic phase

(4) retardation phase

(5) stationary phase.

All these phases are not obligatory in the growth of a culture; any one or several of these phases may be absent. Frequently the acceleration and retardation phases are so short as to be imperceptible. There are three constants used in quantitative growth measurements, the length of the lag phase, the rate of the logarithmic phase or the mean generation time, and the total growth or stationary population of the culture.

There are several methods of determining growth. The basic method of estimating bacterial density is the determination of the dry weight. Indirect chemical methods can be used, for example the estimation of nitrogen.

Fisher and Armstrong (1947), with cultures grown on media which contained an ammonium salt as sole nitrogen source, estimated the decrease of free ammonia in the medium, and found that this correlated well with growth. McIlwain (1941) determined growth by oxygen consumption, and by acid production, which is obviously a very limited method. Van Niel (1944) found centrifugal techniques of great value. The most widely used methods are based on the determination of transmitted or scattered light. It is extremely important with all these indirect methods to express the results in terms of either bacterial density (dry weight) or bacterial concentration (cell numbers). The two methods available for measuring cell numbers are either by viable count, or by total counts using a bacterial haemocytometer.

The Lag Phase

This is the period from the time the inoculum cells are placed in the medium until they proceed to grow and is influenced by several factors. Mueller (1885) reported that the lag phase was affected by the age of the inoculum. He found that the lag phase was shorter with growing cells than with cells from the stationary phase. Rahn (1906) found that the smaller the number of cells in the inoculum, the longer the lag period. Other observations have shown

that the medium, temperature, bacterial strain, age of and number of cells in the inoculum all influence the length of the lag phase.

Lodge and Hinshelwood (1939) found that the lag phase of Bacterium lactis aerogenes (Aerobacter aerogenes) in an artificial medium containing glucose and phosphate buffer was indefinitely lengthened as the concentration of Mg^{++} ions present in the medium was reduced to zero. Thus Mg^{++} ions are necessary for the onset of cell division. Once growth has begun, however, there is no effect on either growth rate or stationary population. On the other hand, these workers and others (Pooh and Hinshelwood, 1940, Monod, 1942) have stressed the adverse effect of traces of heavy metals and other impurities on bacterial growth in simple defined media; indeed at one time Monod (1942) attributed all lag phenomena to the presence of heavy metals, a concept which is not now tenable.

Lodge and Hinshelwood (1943) extended the earlier observations on the effect of the age of the inoculum on the lag phase. They showed that, when a synthetic glucose medium containing asparagine as the source of nitrogen was inoculated with Bact. lactis aerogenes, from a similar medium, the lag period depended on the 'age' of these inoculating cells. The length of lag increased steadily as the inoculum cells aged over the first few days.

When an ammonium salt was used as the nitrogen source the phenomenon which they termed 'early lag' appeared. They found that very young inocula showed a considerable lag, which fell to zero as they aged and then rose again in what may be termed 'late lag'. Gale (1940), Woolridge and Glass (1937) and Woods and Trim (1942) all found that the activities of individual enzymes, studied in washed cell suspensions which were not multiplying, depended upon the age at which the cells were harvested from the growing culture. The activity rose to a maximum and then declined. During the lag phase there must be regeneration of inactivated enzymes.

Lodge and Hinshelwood (1943) found that 'early lag' was completely removed by the addition of sterilized filtrates from fully grown cultures, and suggested that the cells must require some diffusible growth factor, or factors, which is built up to a threshold concentration during the lag phase. With an older inoculum enough of this factor would be transferred to supply the needs of the cells, whereas with young inocula so little is carried over that it must be manufactured by the cells. The oft noted dependence of the length of the lag phase on the size of the inoculum was explained by this theory. The more cells in the inoculum, the quicker the required concentration of the growth factor will be produced, and

vice versa. Lodge and Hinshelwood (1943) also observed that if amino acids were used as sources of nitrogen there was no 'early lag' and no influence of inoculum size. Dagley, Dawes and Morrison (1949) made a study of the response of 'early lag' to various compounds in an attempt to reveal the nature of the intermediates and the reaction sequences occurring before cell division commenced. They found that washed suspensions of cells harvested at the onset of stationary phase, cells which should have exhibited no lag, when used as inocula for glucose ammonium basal medium did exhibit a lag period. Both 'early lag' and the lag produced by washing were partially removed by the addition of various amino acids, most notably glutamate. Fumarate, malate and acetate caused a small response, while succinate produced an effect similar to glutamate. Bact. lactis aerogenes grew almost immediately if 10 parts per million succinic acid were added to the basal medium containing a little pyruvate, even though it was aerated with CO_2-free air.

Dagley and Hinshelwood (1938) found that when cultures of Bact. lactis aerogenes were aerated with a stream of CO_2-free air, growth was delayed indefinitely. This observation had already been made with several other bacteria by Gladstone, Fildes and Richardson (1935).

Elsden (1938) showed that CO_2 was assimilated and was paralleled by the formation of succinic acid, work which has been supported by many other workers. Thus it appeared that during the lag phase, cells synthesize amino acids, mainly glutamate. Dagley, et al (1950a) showed the presence of amino acids in filtrates from Esch. coli and A. aerogenes by chromatography. This work has been extended by Johnson (1953), who found that glutamic acid was the first amino acid to appear in the early logarithmic phase of Esch. coli grown on malate. Lwoff and Monod (1947) found that glutamate, aspartate and closely related compounds were able to replace CO_2 in the metabolism of Esch. coli. Ajl and Werkman (1948) observed that several compounds of the tricarboxylic acid cycle were able to replace CO_2 in the basal medium for A. aerogenes and Esch. coli. Morrison and Hinshelwood (1949) noted that the 'late lag' of Esch. coli cultures could be partially abolished by the addition of glutamate, aspartate and other compounds. Dagley, Dawes and Morrison (1950b) studied the effect caused by each acid of the tricarboxylic acid cycle and found that whereas some caused an acceleration, others caused a retardation in growth.

Lampen, Roepke and Jones (1947) working with a mutant of Esch. coli established that bacteria metabolize sulphur thus:

$\longrightarrow SO_4^{"} \longrightarrow SO_3^{"} \longrightarrow S^{"}$ ⟶ Cysteine ⟶ Cystathionine ⟶ Homocysteine ⟶ Methionine.

Dagley et al (1949) discovered that, in basal medium containing sulphate as the sole source of sulphur, several of the compounds in this sequence reduced 'early lag'.

Phenol is a well known growth inhibitor. Gale and Taylor (1947) showed that in gram-positive bacteria, it owed its bacteriocidal effect to its ability to lyse the cell wall, which allowed the stored amino acids to escape. Dagley, Dawes and Morrison (1950c) showed that the inhibitors phenol, alcohols, acetone and ethyl acetate with a gram-negative organism, A. aerogenes, inhibited cell division and caused a lag period, the duration of which depended on the concentration of the inhibitor. The majority of amino acids reduced this lag, but in the case of ethanol, propanol and phenol, some reduced it while others lengthened it. They concluded that the bacteriostatic action of phenol in this organism was due to its inhibitory effect on the production of metabolites essential for rapid cell division.

Logarithmic Phase.

In this phase the growth rate is constant. It is almost independent of the substrate concentration. When Monod (1942) plotted the growth rate of Esch. coli at

37°C as a function of the concentration of glucose in the medium he found that the maximum growth rate was 1.37, and that the concentration giving half-saturation was 0.5×10^{-4} M glucose. Similar results have been obtained by Penfold and Norris (1912), Dagley and Hinshelwood (1938) and Lodge and Hinshelwood (1939). Caldwell and Hinshelwood (1951) with inorganic phosphate showed that growth occurred down to a concentration as low as 10^{-6} molar. Lodge and Hinshelwood (1939) found that the mean generation time of cultures was not affected by the pH of the medium, and this was confirmed by the findings of Dagley, Dawes and Foster (1953). The effect of toxic products usually rises from negligible proportions to complete inhibition. This ensures that the growth rate over a wide range of concentrations of toxic products is normally unaffected, and that when these start to modify the growth rate, it is with such an effect that there is complete inhibition and the stationary phase is entered (Hinshelwood, 1944).

The Stationary Phase.

The main factors causing cessation of growth in a culture are given as 1) exhaustion of nutrients, 2) accumulation of toxic products and 3) the development of an adverse pH. Lodge and Hinshelwood (1939) carried out a quantitative study of growth, and showed that according to

the circumstances any of these factors may be the limiting one. It is important when using stationary population as a measure of growth to know the limiting factor. These factors do not affect the growth rate of the cultures, but do affect the point at which growth ceases.

An aerated culture supports a larger population than one similar in all respects except that it is unaerated. Hinshelwood (1944) suggested that this was probably due to the removal of toxic products. Dagley et al. (1950b) showed that sterile filtrates from an unaerated culture of A. aerogenes produced growth responses if sufficient oxygen was available. Dagley et al. (1950a) suggested that the supply of oxygen enabled cultures to utilize certain products of metabolism. The sterile filtrates used by Dagley et al. (1950b) were tested at an optimum pH for growth. Dagley et al. (1953) found that the inhibitory effect of formic acid on growth increased as the pH of the medium was lowered. The amount of formic acid produced in an unaerated culture was found to be sufficient to inhibit the growth of a culture at the final pH of the unaerated culture.

Dagley et al. (1950a) demonstrated that, in aerated cultures, the pyruvate concentration rose steadily during growth, increased rapidly just before stationary phase and then fell off after growth had ceased. There was similar

behaviour in unaerated cultures, but the decrease in pyruvate concentration during stationary phase was less pronounced. If however the unaerated stationary phase culture was aerated, there was an overall decrease in the pyruvate concentration and an increase in the stationary population. Bagley, Lawes and Morrison (1951c) found that when an unaerated stationary phase culture of A. aerogenes was aerated there was an immediate increase in pyruvate. When growth recommenced this pyruvate concentration decreased. It rose again while growth continued and decreased finally when the aerated stationary phase was reached. They showed that this pyruvate production was dependent on the presence of glucose. They concluded that the cessation of growth in unaerated cultures when the nutrients were not exhausted was due to the failure of the transfer of hydrogen from reduced DPN to metabolites. This would cause glycolysis to stop at the glyceraldehyde stage and would mean that pyruvate was not produced at a rate adequate for logarithmic growth. Studying the effect of pH on the growth of Esch. coli and A. Aerogenes, Bagley et al. (1953) found that the pH effect was more pronounced in unaerated than in aerated cultures, and suggested that the transfer of hydrogen from reduced DPN to metabolites is inhibited by pH values which do not affect its transfer to oxygen.

Ravin (1952) found that an increase in the buffer concentration of the medium, without a change in its initial pH value, greatly increased the amount of growth of A. aerogenes cultures. A decrease in the initial pH value of the medium decreased the amount of growth. He concluded that the decreased pH values limited the growth, when nutrients were in excess, due to the quicker development of adverse pH values. He criticized the conclusion of Hagley et al (1951c) that since aeration could cause the recommencement of growth, the pH of the medium could not be adverse to growth, and suggested that aeration could cause the resumption of growth due to a fuller utilization of the accumulated organic acids. Hagley et al (1953) allowed three cultures of A. aerogenes at initial pH values, 5.5, 6.5, and 7.5, to grow under unaerated conditions. When the stationary phase was reached these cultures were aerated with a resultant production of pyruvic acid and an increase in population. They found that the pH rose during aeration if the initial pH had been above 6.1, but if the initial pH was between 5.0 and 6.0 then the pH continued to fall after aeration had commenced and while growth was occurring. This clearly refuted Ravin's (1952) claim that growth cessation is due solely to an unfavourable pH value. Furthermore, Hagley,

Lawes and Morrison (1952) have pointed out that the phrase, "adverse pH", has meaning only if it is related to the growth conditions. An unaerated culture of A. aerogenes inoculated into a medium at pH 7.1 stopped growing when the pH reached 5.8. Fresh medium at this pH supported growth. Thus pH 5.8 was only adverse to growth under the conditions prevailing in the medium after it had supported growth from an initial pH of 7.1.

EXPERIMENTAL.

Experimental Methods.

The organism used throughout this work was Escherichia coli, N.C.T.C. 5928. The stock cultures were maintained by monthly subculture on to nutrient agar slopes. Liquid cultures were grown in a glucose-ammonium salt medium containing KH_2PO_4, 5.4 g., $(NH_4)_2SO_4$, 1.8 g., glucose, 12 g., and $MgSO_4.7H_2O$, 0.4 g., per litre. The liquid cultures for use as inocula were grown in 6" x 1" Pyrex tubes containing 25 ml. glucose-ammonium medium at pH 7.1. Cells from an agar slope were always subcultured at least three times in liquid medium before being used for experiments. The buffer used in washed cell suspension experiments contained KH_2PO_4, 9 g. per litre. The pH of the media and buffer solutions was adjusted by the addition of 5N NaOH. All growth, and washed cell suspension, experiments were conducted at 37°C. Glassware was cleaned by boiling in nitric acid (10%) and rinsed in glass-distilled water. Glass-distilled water was used for all media and solutions. In the experiments, cultures were grown in Erlenmeyer flasks, half-filled with medium. Aeration was effected by the passage of a gentle stream of sterile air through the culture.

For the construction of growth curves, the liquid medium was inoculated (4 ml. inoculum per litre) from a

fresh liquid culture at the onset of its stationary phase. 1 ml. samples were withdrawn with a sterile pipette at approximately 40 minute intervals, and 1 drop of formalin added. The bacterial populations were estimated using a Spekker photoelectric absorptiometer with Ilford filters (neutral N 508 and blue OB 2). A calibration curve, relating Spekker drum readings to cell numbers, obtained by a haemocytometer total count was used, and the log (cell number) plotted against time. Under these conditions, the growth curves showed no lag phase, but extrapolated back to the logarithm of the inoculum population at zero time.

Marconi type TF 889 and Muirhead type D 417 A pH meters with glass electrodes were used for pH measurements. Titration curves for the medium were prepared against 5N NaOH and 5N HCl. By reference to these the pH changes were expressed as the amount of acid produced in the medium by the cultures.

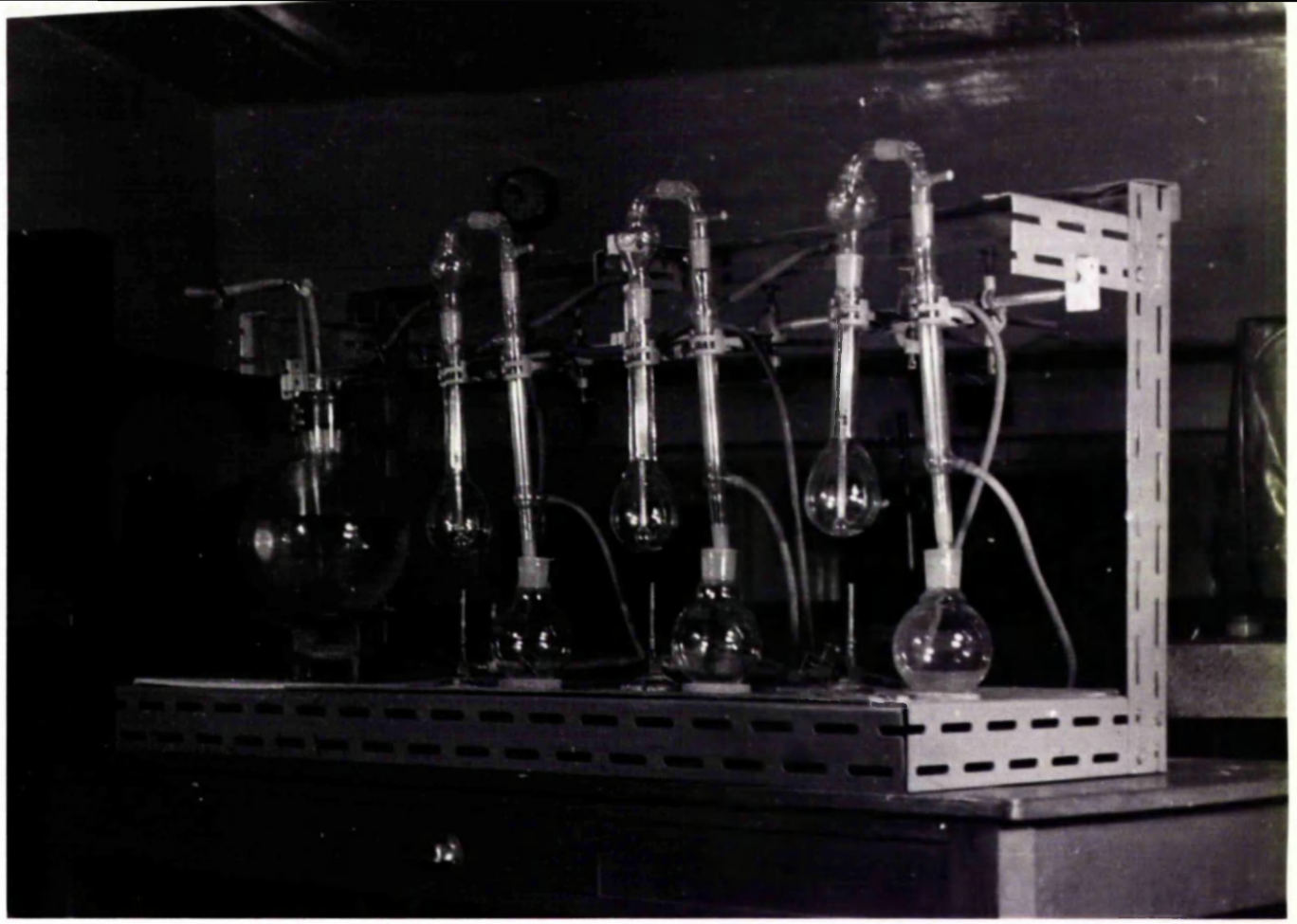
Analytical Methods

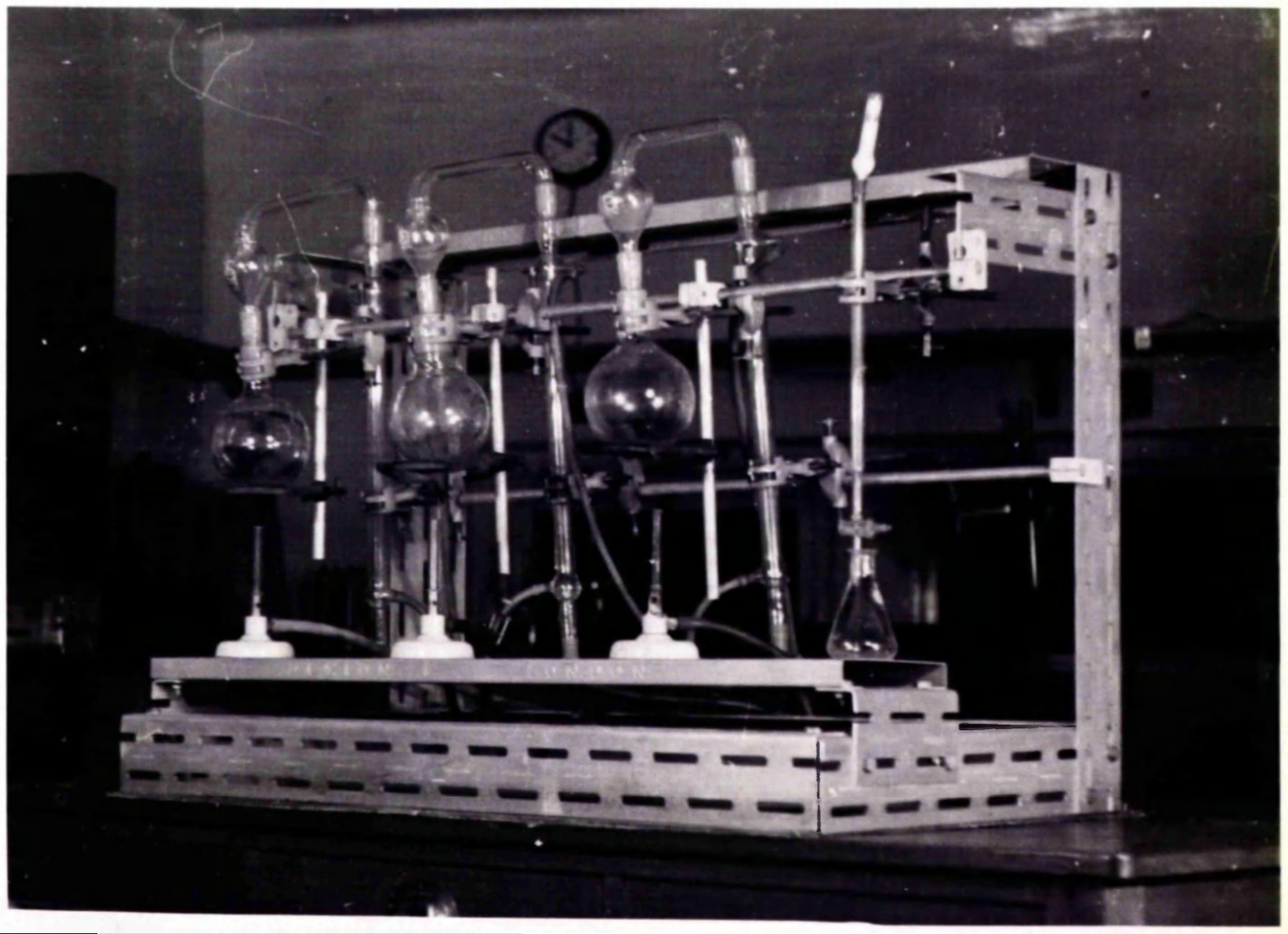
Glucose was estimated by the method of Nelson (1944), a colorimetric modification of the Somogyi reaction (1937). The glucose is allowed to reduce the alkaline copper reagent (containing $CuSO_4.5H_2O$) to cuprous oxide for a controlled time at 100°C. The addition of

an arseno-molybdate reagent to the cooled solution gives a blue colour, which within the range of 0-25 µmoles/ml is proportional to the amount of glucose present. The actual reactions involved in the colour development are uncertain. The different interpretations of the overall reaction have been discussed by Woods and Mellon (1941).

The method of Barker and Summerson (1941) for the estimation of lactic acid was very unsatisfactory, and it was almost impossible to construct a reliable calibration curve. This method depends on the removal of interfering pyruvic acid by a copper calcium complex, the oxidation of lactic acid by conc. H_2SO_4, and the development of a purple colour by the interaction of the acetaldehyde formed with p-hydroxydiphenyl. The two main sources of error are contamination of the tubes by contact with one's hands, which increases the concentration of lactic acid, and excessive oxidation of the lactic acid to acetic acid, which decreases the estimated concentration. Adoption of the technical modifications of Mullen and Noble (1933) and the use of freshly recrystallized p-hydroxydiphenyl, allowed this method to be used with satisfaction.

Pyruvic acid and α-ketoglutaric acid were estimated by the method of Dawes and Holms (1954), which is a modification of the method of Friedemann and Haugen (1943).





The principle is the same in that the total 2,4-dinitrophenylhydrazones are formed, extracted with ethyl acetate, re-extracted with sodium carbonate, and a colour developed with caustic soda. In the modified method, the extraction procedure of Friedemann and Haugen (1943) involving passage of a rapid stream of air or nitrogen is replaced by mechanical shaking in stoppered tubes. Also, all extractions were allowed to stand for 5 minutes after cessation of shaking to permit equilibration of the hydrazones between the two phases. Within 10 to 30 minutes of the colour development, the optical density is read at 500 mμ and 431 mμ on a Unicam S.P. 500 spectrophotometer. The reading at 431 mμ gives the total keto acids present, and from the ratio of the 500/431 mμ readings the ratio of pyruvate to α-ketoglutarate is obtained, and hence the concentrations of the individual keto acids.

Friedemann's (1938) distillation method was used for acetic acid determinations. The apparatus is shown on the preceding page. With this, it was possible to carry out the determinations in groups of three, and to do twelve estimations in the course of one day. The volatile acids are separated in a preliminary steam distillation with acid, tungstate and magnesium sulphate. Formic, pyruvic, and other acids, such as lactic, are removed by redistillation from acidified magnesium sulphate and mercuric oxide.

Constants for Acetic Acid Determinations.

Y / Z	25	50	75	100	250
5	20	10	6.6	5	2
10	10	5	3.3	2.5	1
15	6.6	3.3	2.2	1.66	0.66
20	5	2.5	1.66	1.25	0.5
25	4	2	1.33	1	0.4

Where Y = aliquot titrated (of a 250 ml. distillate).

Z = original volume taken for distillation.

The titre multiplied by the appropriate constant gives acetic acid in μmoles/ml.

Aeration with CO_2-free air, obtained by passing compressed air through a 4' x 3" column of soda lime, at room temperature just before titration with 0.01N NaOH, using phenolphthalein as indicator, quantitatively removes CO_2 without loss of volatile acid. The titre was converted by the equation:

$$\text{Concentration of acetic acid} = \frac{x \times 0.15}{y \times z \times 60} \times 10^6$$

where x = titre of 0.01N NaOH

y = aliquot, from second distillate which has been adjusted to a volume of 250 ml., used for titration.

z = aliquot of original solution used for distillation.

In actual practice a table of constants for several values of y and z was constructed, and the titre multiplied by the appropriate constant (see opposite).

The volatile acids were estimated by the method described by Werkman (1942). Separation of the volatile acids is effected by steam distillation after acidification with H_2SO_4 to pH 2.0. It is necessary to collect twelve volumes of distillate, in order to remove the volatile acids quantitatively. The acid in the distillate is determined by titration with 0.01N NaOH. It was found advisable, if the samples had been stored in the deep

freeze for any length of time, to aerate them with CO_2-free air before distillation.

The determination of succinic acid was carried out by the enzymatic manometric method of Weil-Malherbe (1937). Succinic dehydrogenase was prepared from ox heart. The succinic acid was not extracted with ether since this is only necessary in the presence of α-ketoglutarate, and our results showed that this was not present in the samples used. A suspension of the enzyme is placed in the side-arm of a Warburg manometric flask. A neutral solution of succinic acid, and cresyl blue are placed in the main compartment, and alkali in the centre well. The oxygen uptake gives a quantitative measure of the succinic acid.

Ethanol was estimated by the microdiffusion technique of MacLeod (1949) which depends on the volatility of alcohol and its power to reduce oxidizing agents. The ethanol solution is placed with a saturated solution of potassium carbonate in the outer compartment of a Conway unit. The centre compartment contains alkaline permanganate and the dish is sealed with a cover slide coated with water-glass. The dishes are placed in a dark cupboard for no less than three, and no more than twenty-four hours. The lid is then removed, barium manganate precipitated by the addition of barium chloride, and the remaining permanganate titrated with the reducing agent,

thiourea. The end-point of this titration is reported to be a sudden change of colour of the precipitated barium manganate from steel blue to dark green. Such a colour change did not occur in the majority of our titrations: the barium manganate remained steel blue even after the addition of a considerable excess of reducing agent. A personal communication from Dr. MacLeod explained this finding. The colour change depends entirely on the particle size of the barium manganate precipitate, and if, as appeared to be the case in our titrations, the precipitate is very finely dispersed, it would not be obvious. The disappearance of the last trace of pink colour from the solution was adopted as the end-point. This enabled reproducible results to be obtained with an accuracy of ± 1 µmole ethanol/ml. When the microdiffusion method of Northrup, Ashe and Senior (1919) was used the recovery of ethanol was only 50%. One other modification of the original method was adopted. The alkaline permanganate stored in a brown glass bottle in a dark cupboard was reported to be fairly stable and therefore usable for about two weeks after its preparation. After this time it has deteriorated too much to give reliable titres. This is more or less true, but as the solution slowly deteriorates during these two weeks, so there is a steady deterioration in the titrations. It is no inconvenience to prepare

fresh permanganate for each batch of estimations, and in practice this means that the titrations are more easily performed and give more reliable results.

INSULIN.

Table 1.

Metabolic products of Escherichia coli appearing in medium during aerated growth in glucose-ammonium salt cultures at different initial pH values.

Escherichia Coli I. Aerated

Time after Inoculation (min.)	Log Cell Numbers (10^6/ml.)	pH	(1) Pyruvic Acid (µmoles/l.)	(2) Lactic Acid (µmoles/l.)
180	1.0	5.45	0	27
244	1.64	5.15	0	27
280	2.0	5.1	0	27
330	2.24	5.1	0	27
390	2.59	4.6	27	44
450	2.75	4.1	152	144
523	2.79	3.96	70	174
576	2.81	3.96	63	55
180	1.0	6.45	0	85
244	1.69	6.4	0	72
280	2.18	6.35	0	--
330	2.42	6.25	0	100
390	2.69	5.95	136	205
450	2.99	5.25	815	444
523	3.14	4.9	1,400	1,711
576	3.14	4.8	1,660	2,044
180	1.0	7.4	0	44
241	1.54	7.35	0	22
280	-	7.2	0	44
330	2.26	7.2	0	27
390	2.64	7.05	0	44
450	2.87	6.65	15	20
523	3.27	5.55	1,000	1,433
576	3.31	5.1	1,720	2,966
610	3.31	5.0	2,060	2,766

Growth

(3) Total Volatile Acid (µmoles/l.)	(4) Acetic Acid (µmoles/l.)	(5) Formic Acid (3)-(4) (µmoles/l.)	(6) Ethanol (µmoles/l.)
800	800	0	0
900	600	300	0
1,000	600	400	0
1,000	-	-	0
1,300	600	700	0
1,900	2,000	0	0
2,900	2,600	300	0
-	-	-	0
860	0	860	0
1,200	600	600	0
1,530	900	630	0
1,860	1,000	860	0
5,260	2,600	2,660	1,050
8,000	3,900	4,100	2,380
9,760	4,400	5,360	3,160
8,430	4,100	4,330	1,700
860	200	660	1,360
1,000	500	500	1,700
1,000	600	400	1,700
1,460	800	660	1,360
2,400	1,600	800	1,360
8,100	4,500	3,600	2,040
17,460	8,300	9,160	3,740
15,900	9,100	6,800	3,400
15,700	8,200	7,500	340

Table 2.

Metabolic products of Escherichia coli appearing in medium during unaerated growth in glucose-ammonium salt cultures at different initial pH values.

Escherichia Coli, II Unaerated

Time after Inoculation (min.)	Log Cell Number (10^6/ml.)	pH	(1) Pyruvic Acid (µmoles/l.)	(2) Lactic Acid (µmoles/l.)
180	1.28	5.35	0	0
222	1.49	5.25	0	0
267	1.94	5.15	52	0
310	2.16	4.9	80	0
353	2.21	4.7	111	0
413	2.34	4.4	167	29
482	2.42	4.25	150	155
576	2.45	4.15	176	230
180	1.64	6.4	0	0
222	1.84	6.4	0	0
267	2.03	6.3	0	0
310	2.30	6.2	30	0
353	2.51	6.1	-	171
413	2.72	5.65	180	-
482	2.85	5.1	430	711
576	2.95	4.8	760	1,000
180	1.39	7.35	0	44
222	1.54	7.3	0	44
267	1.74	7.2	0	44
310	2.0	7.2	0	44
353	2.19	7.2	0	127
413	2.49	6.9	13	177
482	2.79	6.55	75	284
576	3.07	5.45	530	1,233
610	3.07	5.1	1,120	2,000

Growth

(3)	(4)	(5)	(6)
Total Volatile Acid (µmoles/l.)	Acetic Acid (µmoles/l.)	Formic Acid (3)-(4) (µmoles/l.)	Ethanol (µmoles/l.)
830	400	430	0
900	–	–	0
940	800	140	0
1,170	–	–	0
1,610	600	1,010	0
1,960	1,200	760	0
2,000	1,000	1,000	1,280
2,600	1,800	800	4,640
–	1,200	–	0
1,200	800	400	0
1,200	600	600	0
2,400	1,400	1,000	0
3,400	1,800	1,600	0
7,800	2,600	5,200	0
8,860	3,400	5,460	0
9,800	5,400	4,400	0
800	200	600	1,300
930	800	130	2,650
1,000	800	200	2,280
1,230	600	630	–
1,900	1,200	700	4,180
–	2,200	–	4,770
9,900	4,400	5,460	4,540
14,800	7,200	7,600	6,650
16,960	9,000	7,960	8,740

Table 5.

Correlation between monocarboxylic acids produced by Escherichia coli during growth in glucose-ammonium salt medium and calculated total acid production from observed pH changes.

Arrows indicate stationary phase.

	Aerated Growth			
Initial pH	pH of Sample	(1) Total * Acid formed (equiv./l.)	(2) Total + Monocarboxylic Acid formed (equiv./l.)	Acid ++ = (1)-(2) (equiv./l.)
5.5	5.45	0.15	0.53	-0.6
	5.15	0.75	0.93	-0.18
	5.1	1.0	1.37	-0.37
	5.1	1.0	1.37	-0.37
	4.8	2.0	1.50	0.32
	4.1 →	3.25	2.5	0.95
	3.95	3.75	3.16	0.59
	3.95	3.75	4.0	-0.25
6.5	6.45	0.5	0.94	-0.41
	6.4	1.25	1.23	-0.03
	6.35	2.0	1.57	0.43
	6.25	3.75	1.96	1.79
	6.35	6.75	5.59	1.16
	6.25 →	9.9	9.26	0.64
	4.9	10.75	12.87	-1.12
	4.8	10.99	12.13	-1.13
7.5	7.4	3.25	0.9	2.35
	7.35	4.0	1.02	2.98
	7.2	6.5	1.02	5.48
	7.2	6.5	1.5	5.0
	7.05	11.25	2.42	8.81
	6.35	21.25	8.53	12.72
	6.55 →	32.75	19.99	12.76
	6.1	34.0	20.58	13.42
	6.0	34.25	20.52	13.73

*Calculated from observed pH change and titration curve of medium

+Sum of total volatile, lactic and pyruvic acid determined by culture.

++Assumed dicarboxylic acid present: (μmoles/l.) obtained by dividing this value by 2.

Unaerated Growth

pH of Sample	(3) Total $^{+}$ Acid formed (μequiv./l.)	(4) Total $^{+}$ Monocarboxylic Acid formed (μequiv./l.)	Acid $^{++}$ = (3)-(4) (μequiv./l.)
5.35	0.5	0.93	-0.38
5.25	0.65	0.90	-0.25
5.15	0.9	1.06	-0.15
4.9	1.5	1.27	0.23
4.7	1.75	1.73	0.02
→ 4.4	2.5	2.18	0.32
4.25	5.0	-	-
4.15	3.25	3.03	0.22
6.4	1.15	-	-
6.4	1.15	1.26	-0.11
6.3	2.75	1.2	1.55
6.2	4.25	2.43	1.82
6.1	5.25	3.57	1.68
6.05	8.75	7.97	0.78
→ 5.1	10.2	10.02	0.18
4.8	10.9	11.56	-0.66
7.35	4.25	0.84	3.41
7.3	5.0	0.94	4.06
7.2	7.5	1.1	6.4
7.2	7.5	1.27	6.23
7.2	7.5	2.02	5.48
6.9	15.0	-	-
6.55	22.5	10.24	12.26
→ 5.45	33.15	16.56	16.59
5.1	34.0	20.14	13.86

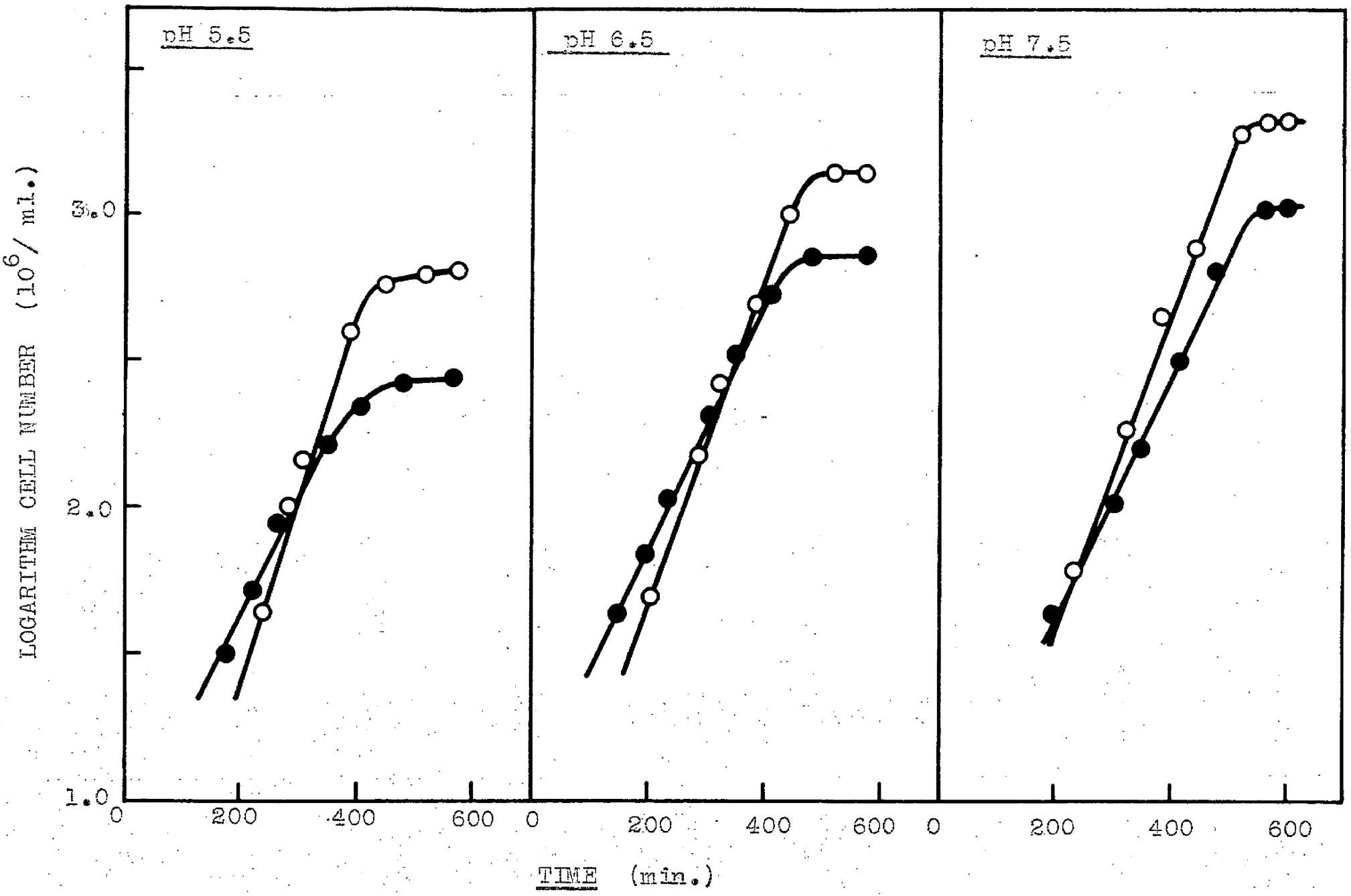

pH 5.5
pH 6.5
pH 7.5
LOGARITHM CELL NUMBER (10^6/ml.)
3.0
2.0
1.0
0
200
400
600
TIME (min.)

Fig.1. Growth curves of *Escherichia coli* grown in glucose-ammonium salt media of initial pH values 5.5, 6.5 and 7.5, ● unaerated, o aerated.

Fig.2a. Production of total volatile acids during unaerated and aerated growth of Escherichia coli in glucose-ammonium salt media at initial pH values ○ 5.5, □ 6.5 and △ 7.5. Solid symbols indicate samples taken after the onset of the stationary phase.

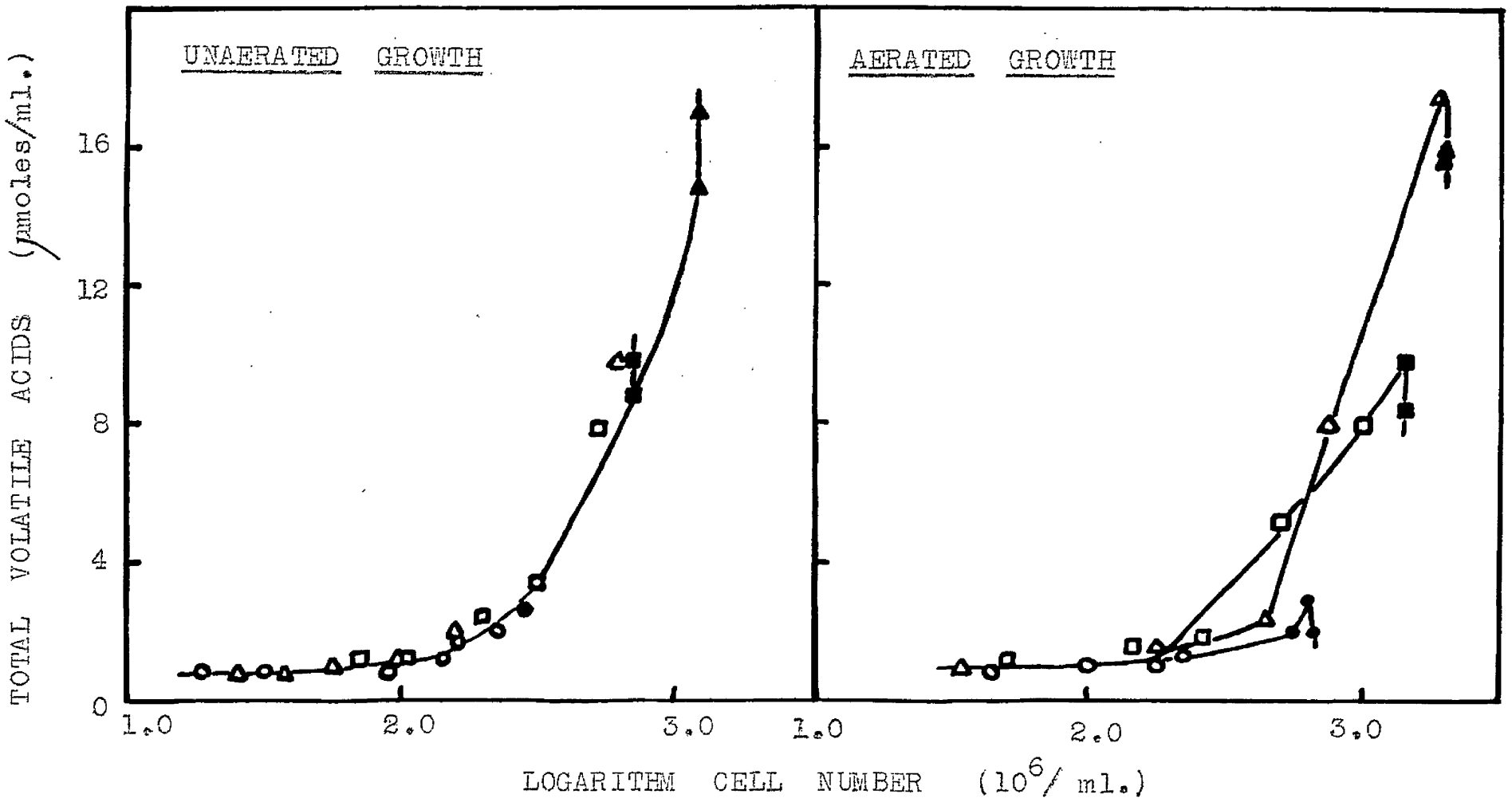

UNAERATED GROWTH
AERATED GROWTH
TOTAL VOLATILE ACIDS (μmoles/ml.)
0
4
8
12
16
1.0
2.0
3.0
LOGARITHM CELL NUMBER (10^6/ ml.)

Fig. 2b. Production of pyruvic acid during unaerated and aerated growth of Escherichia coli in glucose-ammonium salt media at initial pH values ○ 5.5, □ 6.5 and △ 7.5. Solid symbols indicate samples taken after the onset of the stationary phase.

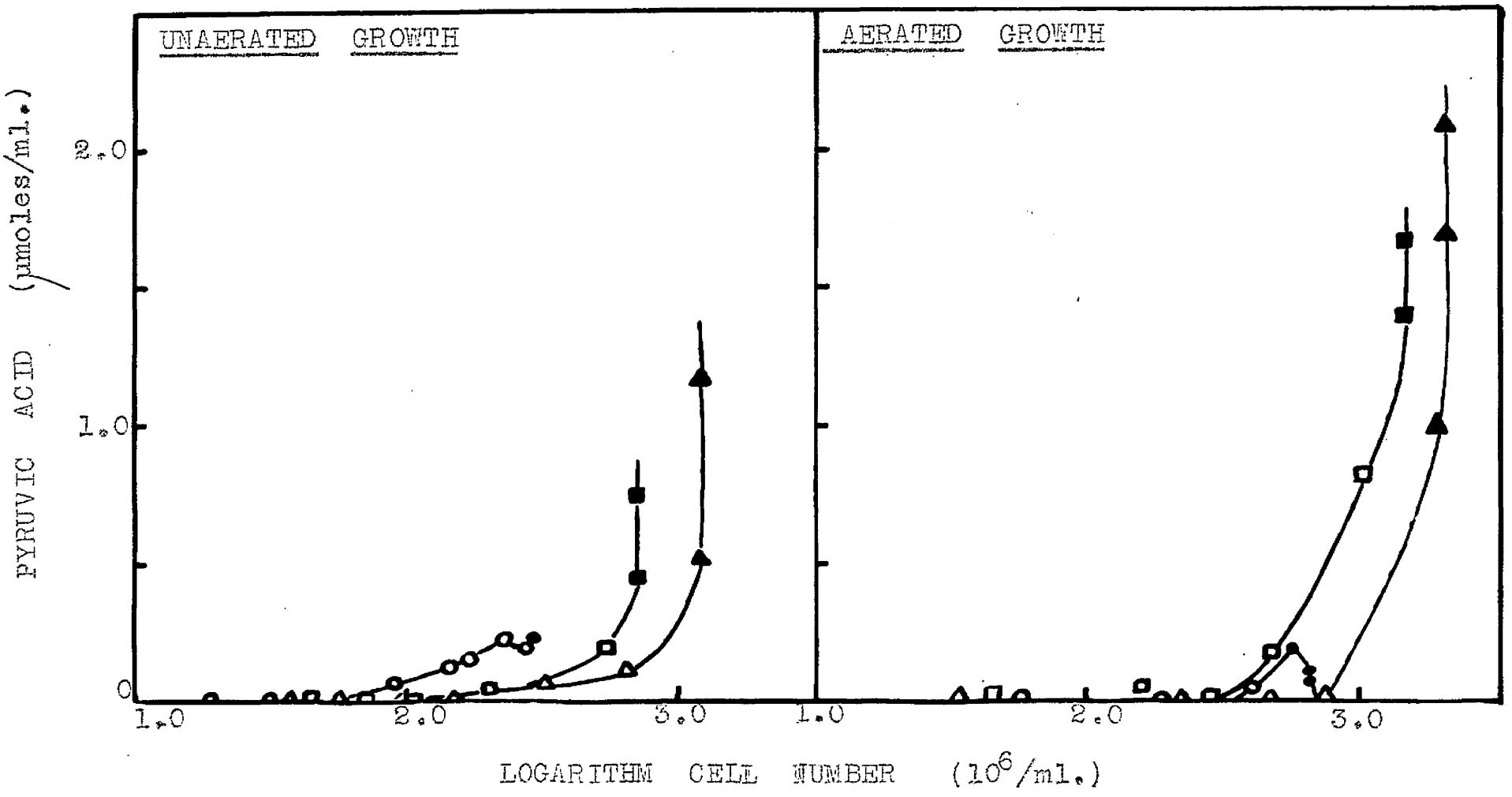

UNAERATED GROWTH
AERATED GROWTH
PYRUVIC ACID (μmoles/ml.)
2.0
1.0
0
1.0
2.0
3.0
1.0
2.0
3.0
LOGARITHM CELL NUMBER (10⁶/ml.)

Fig. 2c. Production of lactic acid during unaerated and aerated growth of Escherichia coli in glucose-ammonium salt media at initial pH values ○ 5.5, □ 6.5 and Δ 7.5. Solid symbols indicate samples taken after the onset of the stationary phase.

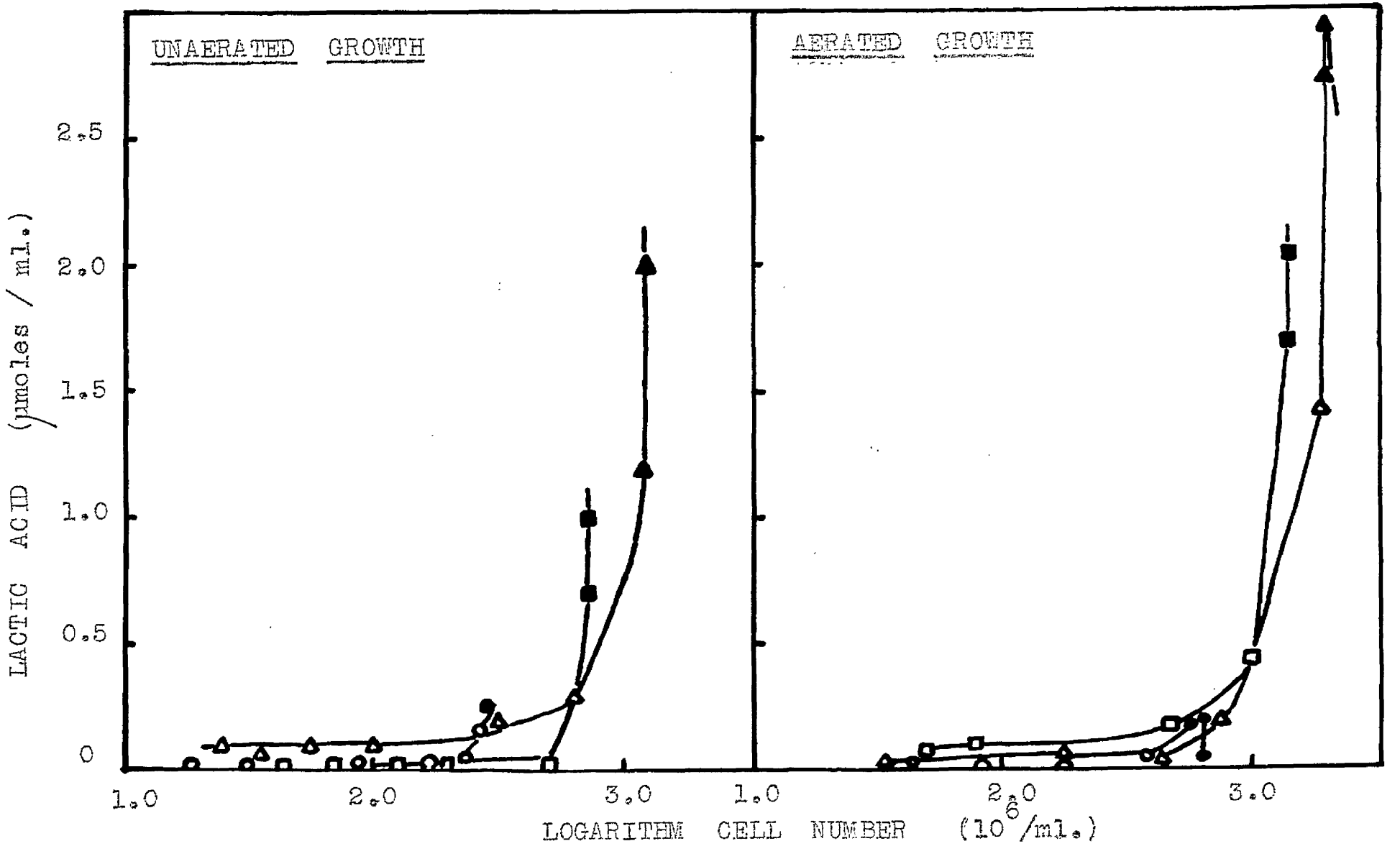
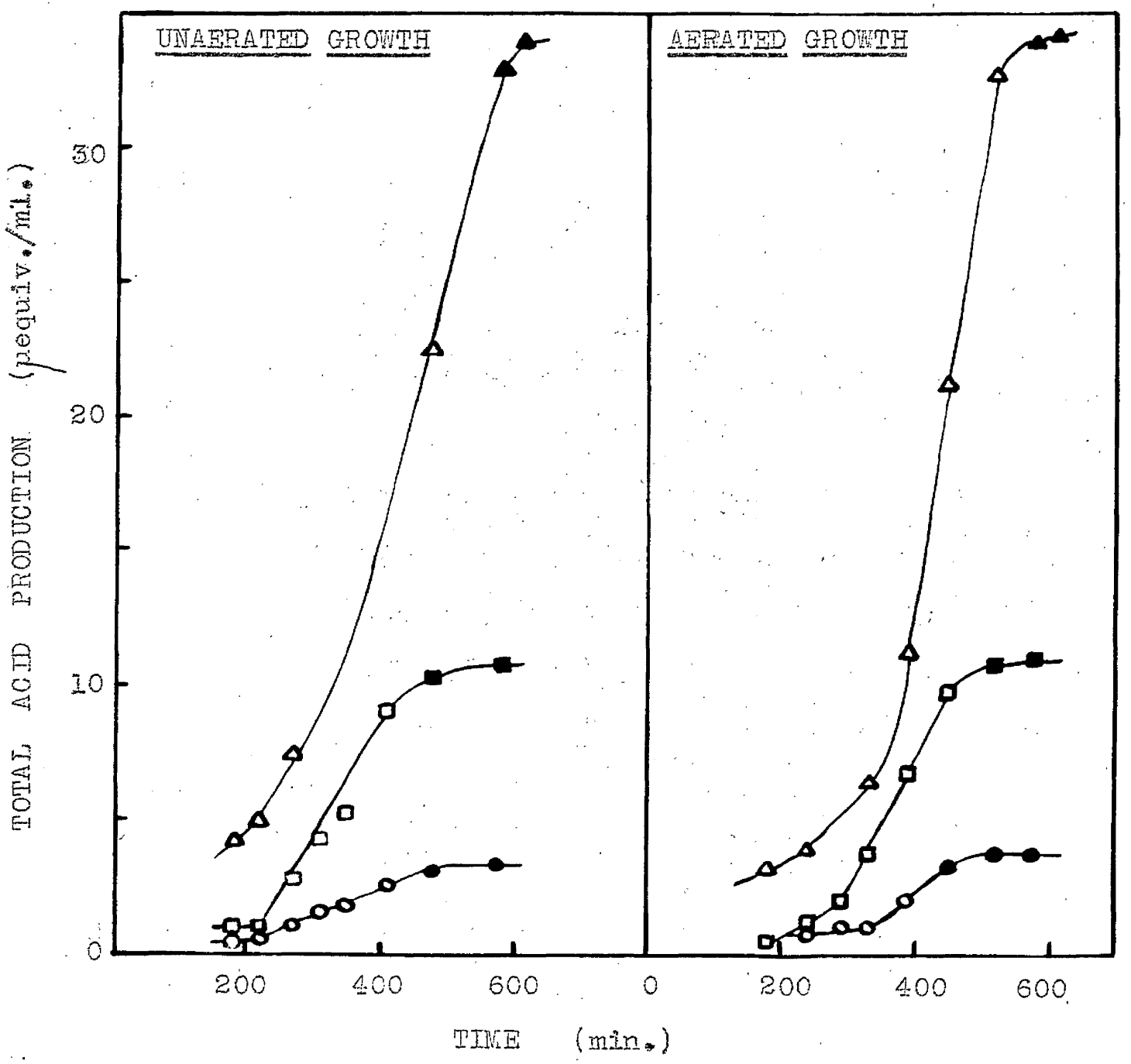

UNAERATED GROWTH
AERATED GROWTH
LACTIC ACID (μmoles / ml.)
2.5
2.0
1.5
1.0
0.5
0
1.0
2.0
3.0
1.0
2.0
3.0
LOGARITHM CELL NUMBER (10^6/ml.)

Fig.20. Total acid production, calculated from changes in pH, during unaerated and aerated growth of Escherichia coli in glucose-ammonium salt media at initial pH values ○ 6.5, □ 6.9 and △ 7.5. Solid symbols indicate samples taken after the onset of the stationary phase.


UNAERATED GROWTH
AERATED GROWTH
TOTAL ACID PRODUCTION (μequiv./ml.)
30
20
10
0
200
400
600
0
200
400
600
TIME (min.)

Six 1 l. Erlenmeyer flasks containing 500 ml. glucose-ammonium salt medium were prepared. Three of these, with initial pH values 5.5, 6.5 and 7.5 respectively were aerated. The other three, with the same initial pH values, were left without aeration. They were all inoculated with 2.0 ml. of stationary phase Esch. coli culture and growth followed. Growth curves were constructed and are shown in Fig.1. Samples withdrawn at regular time intervals were centrifuged and the pH and acetic, total volatile, total keto, pyruvic, α-keto-glutaric, lactic acid and ethanol concentrations of the supernatants determined. These results are given in Tables 1 and 2, and some are shown graphically in Fig.2a, b, c and d. There was a minute concentration, which never exceeded 0.05 μmoles/ml., of α-ketoglutaric acid present in the cultures during the early stages of growth but this disappeared before the end of the logarithmic phase of growth. Pyruvic acid can, therefore, be regarded as the total keto acid present.

When the theoretical acid production, calculated from the pH changes, was compared with the total estimated acid production as given by the sum of the concentrations of the monocarboxylic acids, Table 3, it was observed that there was fairly good agreement for the cultures with initial pH values of 5.5 and 6.5 under both aerated and

unaerated conditions. For both the cultures with initial pH value 7.5, there was a large difference between the two values. Two possible reasons for this were investigated. It was thought that the more alkaline cultures might hold a higher concentration of dissolved CO_2 than the more acidic ones. Therefore, the pH value of fresh supernatants from three unaerated cultures of initial pH values 5.5, 6.5 and 7.5 were determined before and after the dissolved gases had been removed by means of a vacuum pump. There was a very slight rise in the pH value, equivalent to less than 2 μmoles/ml., after the gases had been removed. This could not account for the difference of about 14 μequivalents/ml. acid noted in the pH 7.5 cultures.

As the monocarboxylic acid content of the cultures (pH 5.5 and 6.5) is almost equivalent to the theoretical acid content, there cannot be a high concentration of dicarboxylic acids present. It was possible, however, that dicarboxylic acids might be produced in the cultures with initial pH 7.5 in amounts which could account for the difference between the theoretical and monocarboxylic acid content. Harden (1901) found traces of succinic acid in cultures of Bacillus coli communis. Therefore, the amount of succinic acid present in the supernatants

Table 4.

Utilization of glucose by, and change in pH value of medium in, stationary phase cultures of Escherichia coli grown in glucose-ammonium salt medium at different initial pH values, with and without aeration. (Arrows indicate stationary phase).

Initial pH		Unaerated				Aerated			
5.5	Hrs. after inoculation	6.0 ↓	8.5	11.5	14.0	7.3 ↓	9.8	12.8	15.3
	Glucose concentration (µmoles/ml.)	62.5	62.5	62.5	62.5	62.0	61.25	61.25	65.0
	pH of samples	4.22	4.35	4.25	4.25	4.35	4.35	4.15	4.15
6.5	Hrs. after inoculation	6.8 ↓	9.1	12.8	15.3	7.3 ↓	9.8	12.8	15.3
	Glucose concentration (µmoles/ml.)	62.5	61.25	62.0	62.5	62.5	61.25	60.0	60.0
	pH of samples	5.22	4.8	4.4	4.2	5.0	5.0	4.5	4.5
7.5	Hrs. after inoculation	7.3 ↓	9.8	12.8	15.3	8.1 ↓	10.6	13.0	16.1
	Glucose concentration (µmoles/ml.)	-	53.75	50.0	48.0	55.0	55.0	45.0	42.5
	pH of samples	-	5.32	5.1	4.9	5.85	5.6	5.0	5.08

from the three unaerated cultures was estimated. There was no succinic acid detected in the pH 5.5 and 6.5 cultures, while the concentration in the pH 7.5 cultures was never more than 2 μmoles/ml.

From Tables 1 and 2 it can be seen that there was very little production of metabolites in the early logarithmic growth of cultures. In the late logarithmic phase there was a sudden increase in metabolite concentrations. The acid production, as indicated by the pH changes, levelled off once the cultures had entered the stationary phase (Table 3 and Fig.2d). Bagley et al. (1953) also found for unaerated cultures that the pH fell during growth, but remained unchanged in the stationary phase. This suggested that the non-proliferating cells, if they were no longer producing acid, were no longer utilizing glucose. The rate of glucose disappearance in the stationary phase of Esch. coli cultures was investigated.

The cultures were set up exactly as in the previous experiment. Growth was followed to determine the onset of the stationary phase. Samples were withdrawn every few hours, and the pH and glucose content of the supernatants determined. The results are given in Table 4. Aerated and unaerated cultures of Esch. coli with initial pH values 5.5 and 6.5 did not continue to utilize

the excess glucose in the medium in their stationary phase. Unaerated and aerated cultures with an initial pH value of 7.5 continued to utilize glucose but only for a limited time. Further samples showed that the glucose concentration remained at the levels reached at 15.3 and 16.1 hours respectively.

The cessation of glucose metabolism in stationary phase cultures with initial pH values 5.5 and 6.5 might be due to one or more of three factors, the pH of the cultures at the onset of stationary phase, a high concentration of toxic products in the medium, or some factor affecting the cell wall, which might be removed by washing the cells. The next experiment was designed to test these possibilities. Four Erlenmeyer flasks (250 ml.) containing 125 ml. glucose-ammonium salt medium, initial pH 5.5, and another four, initial pH 6.5, were inoculated and growth followed under unaerated conditions. The glucose-ammonium salt medium used in this experiment contained only 9 g. instead of the normal 12 g. glucose/l. At the onset of the stationary phase the following procedure was carried out. One culture of each pH value was left as it was; another was centrifuged, the cells washed and then resuspended in the original culture filtrate; the third was centrifuged and the cells suspended in the

Table 5.

Utilization of glucose during unaerated growth of Escherichia coli in glucose-ammonium salt medium at different initial pH values: and by the non-proliferating cells in the stationary phase under four different conditions.

Initial pH	Glucose Concentration (µmoles/ml.)			Treatment	Glucose Concentration (µmoles/ml.)					
	Initial	315 min.	360 min.		Initial	60 min.	120 min.	180 min.	240 min.	17 hrs.
5.5	51.25	49.35	45.6	A	45.0	45.0	46.9	45.6	45.6	45.0
	50.0	48.1	46.2	B	45.6	46.2	46.2	45.6	46.2	46.6
	51.2	49.4	48.7	C	45.0	45.6	43.1	43.1	45.6	43.8
	51.2	49.4	49.7	D	46.2	45.6	46.2	46.2	45.7	44.9
		375	420							
6.5	51.8	48.1	45.6	A	45.6	45.6	46.6	42.5	42.5	39.4
	52.4	48.1	43.7	B	40.6	40.6	37.5	36.2	39.0	36.2
	52.4	48.1	43.7	C	45.6	42.5	42.5	40.6	40.6	40.6
	52.4	48.1	45.6	D	46.9	45.6	43.9	43.1	42.5	42.5

Treatment: A = left in medium.
B = centrifuged, washed and replaced in medium.
C = centrifuged, resuspended in fresh medium.
D = centrifuged, washed and resuspended in fresh medium.

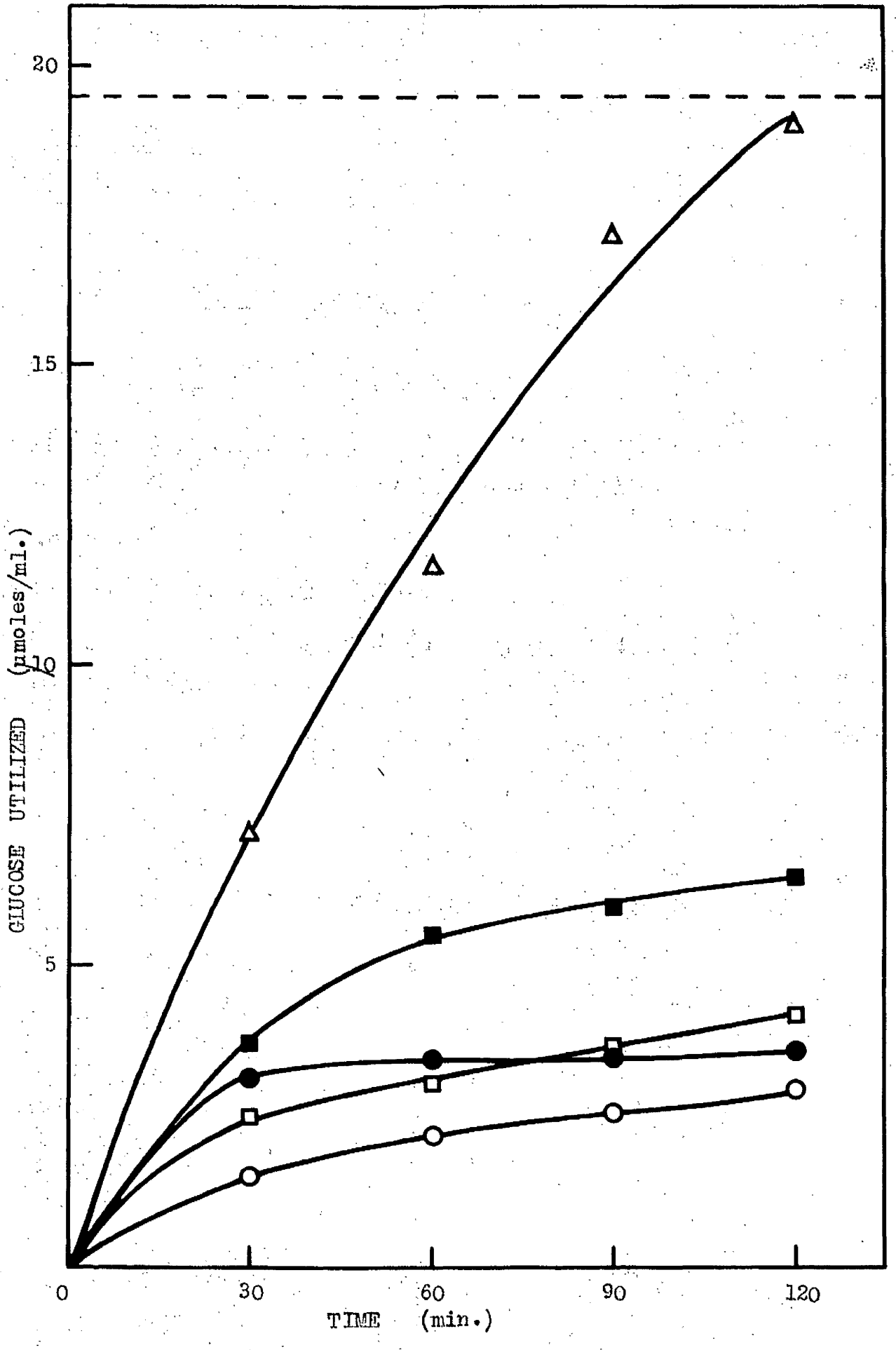
the same volume of new medium as there was supernatant; the last culture of each set was centrifuged and the cells washed and suspended in new medium. The cells were washed with phosphate buffer adjusted to the final pH of the growth media. The new medium for the pH 5.5 cells contained KH_2PO_4 5.4 g., $(NH_4)_2SO_4$ 1.2 g., $MgSO_4.7H_2O$ 0.4 g. and glucose 21.91 g. per litre, adjusted to pH 4.7. The new medium for the pH 6.5 cells contained KH_2PO_4 5.4 g., $(NH_4)_2SO_4$ 1.2 g., $MgSO_4.7H_2O$ 0.4 g. and glucose 20.5 g. per litre, and was adjusted to pH 5.1. The cultures were replaced in the incubator at 37°, and samples removed hourly for glucose determinations. The results are presented in Table 5. During growth, the glucose utilized by the pH 5.5 cultures was 1.9 µmoles/ml. and by the pH 6.5 cultures was 4.3 µmoles/ml. These figures compare favourably with those reported by Foster (1952) which gave the expected utilization by a pH 5.5 culture of Esch. coli as 2.5 µmoles/ml. and by a pH 6.5 culture as 4.5 µmoles/ml.

In the four pH 5.5 cultures there was some glucose utilization and the four different treatments had little or no effect on this. In the four pH 5.5 cultures there was no utilization if the culture filtrates were used and where the cells were placed in fresh medium the glucose utilized was as little as 1.2 and 1.3 µmoles/ml.

The reliability and significance of such low figures are questionable. This it would appear that the inability of stationary cultures of Esch. coli to utilize glucose is not due to toxic products in the medium and, if it is the cells themselves which are affected, this cannot be remedied by washing. The following experiment was carried out to test the effect of pH on the ability of cells to utilize glucose. Tikka (1935) and Stokes (1949) had studied the effect of pH on the products of glucose metabolism. The lowest pH values investigated were 6.4 and 5.62 respectively. The stationary phase pH values of cultures inoculated at pH 5.5 and 6.5 were found in this work to be 4.6 and 5.1. Washed suspensions of cells grown in glucose-ammonium salt medium, pH 7.1, were prepared. The buffering capacity of this medium was increased by using 16.2 g. instead of 5.4 g. per litre KH_2PO_4: the final pH of the culture was still 5.4. The cell suspensions, in phosphate buffer of five different pH values, 7.1, 6.0, 5.6, 5.1 and 4.6 were set up in tubes as shown:

	ml.
Phosphate buffer of appropriate pH	12
Glucose (final concentration 20 µmoles/ml.)	8
Water	1
Cell suspension (28 mg. dry wt./ml.)	4
$MgSO_4.7H_2O$ (10%)	0.1

Fig.6. Utilization of glucose by non-proliferating cell suspensions of Escherichia coli at different initial pH values, ○ 4.6, ● 5.1, □ 5.6, ■ 6.0 and △ 7.1. Dotted line indicates maximum possible utilization.


20
15
10
5
0
30
60
90
120
GLUCOSE UTILIZED (µmoles/ml.)
TIME (min.)

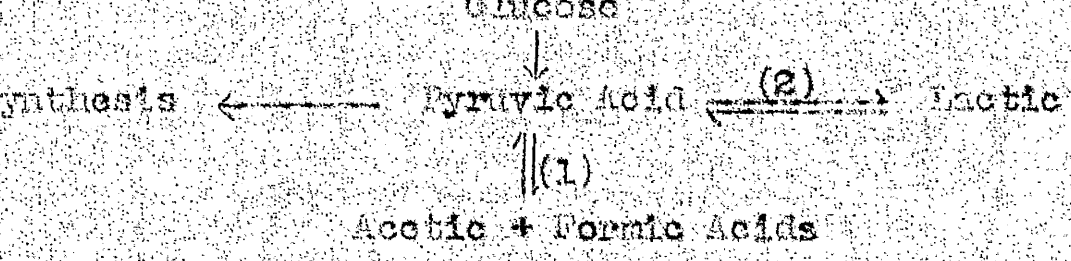
The results, expressed as glucose utilization, are plotted in Fig.3.

DISCUSSION.

Eagley et al. (1950a) and (1951c) found that there was very slight production of pyruvic acid during logarithmic growth of A. aerogenes and Esch. coli. The concentration increased rapidly just before the onset of the stationary phase and then decreased in the non-proliferating cultures. This decrease was more rapid in aerated than in unaerated cultures. Fig.2b shows that there was very little production of pyruvate during the early logarithmic phase, and an increase just before the onset of stationary phase. The pyruvate concentration continued to increase in this experiment, and also in a duplicate one, in the stationary phase cultures at the two pH values 6.5 and 7.5 but not at 5.5. In the aerated pH 5.5 cultures the pyruvate concentration had almost disappeared within 100 minutes of the onset of stationary phase. Pyruvic acid is a 'building block' in cellular synthesis. It would appear that during the early logarithmic growth phase its utilization keeps pace with its production. As the cultures approach their stationary phase, production must outstrip utilization causing an accumulation of pyruvate in the medium.

This pattern is repeated in the case of all the monocarboxylic acid end products of glucose metabolism (Fig. 2a and c). Total volatile acids, acetic acid and

lactic acid are all present in very small concentration in the early stages of growth, and increase suddenly before the onset of the stationary phase. The increase in lactic acid concentration coincides with that of pyruvic acid. The increase in concentration of the volatile acids precedes that of pyruvic acid.



When pyruvic acid utilization for growth purposes begins to decrease, and pyruvic acid begins to accumulate, it must undergo phosphoroclastic fission, reaction (1), to acetic and formic acids, causing an initial accumulation of total volatile acid. For every mole of pyruvic acid undergoing phosphoroclastic fission, two moles of acid are formed, one of acetic and one of formic. This increase in the acid content of the medium will cause a decrease in the pH. Bagley et al. (1955) found that the pH of the glucose-ammonium salt medium fell very slowly during the early logarithmic phase of growth, then decreased rapidly just before the onset of the stationary phase. This lowering of the pH, effected initially by

the phosphoroclastic fission of pyruvate, will suppress this reaction and will favour the formation of lactic acid by lactic dehydrogenase, reaction (2) (Pikka, 1955). The increase in lactic acid concentration is paralleled by an accumulation of pyruvate. The concentrations of these two acids always show a great similarity. An example of the interrelation of lactic and pyruvic acids is shown in Fig.2b and c. In the pH 5.5 aerated culture, where the pyruvate concentration almost disappeared in the stationary phase, lactic acid showed an identical decrease.

There is remarkable similarity between the concentrations of volatile acid produced in the unaerated and aerated cultures shown both by the total volatile acids (Fig.2a) and by acetic acid (Tables 1 and 2). Pyruvic acid reaches a higher concentration in aerated than in unaerated cultures at pH 6.5 and 7.5. This is also true of lactic acid. It is rather surprising to find a higher concentration of a reduced end-product in a culture with a better supply of oxygen.

These results (Tables 1 and 2) show that figures quoted for the metabolic products of a growing culture will be very different from those quoted for a stationary phase culture. In the aerated pH 6.5 culture, the ethanol concentration was 3,600 μmoles per litre at the beginning of stationary phase. This had dropped to 1,700 μmoles

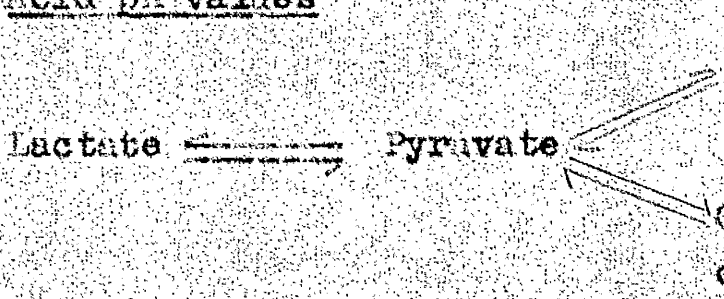
per litre within 55 minutes. In the aerated pH 7.5 culture, the ethanol concentration decreased from 3,400 µmoles per litre at the onset of stationary phase to 340 µmoles per litre after 34 minutes. In the unaerated cultures, 1,200 µmoles per litre of ethanol at stationary phase increased to 4,640 µmoles per litre in 94 minutes at pH 5.5, while at pH 7.5 a concentration of 3,650 µmoles per litre increased to 8,740 µmoles per litre in 34 minutes. Thus the supernatant of a culture, which has been incubated for five days when growth must have ceased after about 24 hours (Scheffer, 1928), will not give a true picture of the metabolites produced during active growth if, once the cells have entered the stationary phase, 34 minutes can bring an increase of 2,000 or a decrease of 3,000 µmoles per litre in the concentration of an end-product.

The results of total volatile acid concentrations in unaerated cultures at the three different initial pH values, when plotted against the logarithm of cell number (Fig.2a), show remarkable similarity between the three cultures. During logarithmic growth the same number of cells, irrespective of the initial pH of the medium, produced the same amount of metabolites. Within the limits of experimental error this same similarity can be seen for all the metabolites in the three cultures at different pH values, under both aerated and unaerated conditions.

Although different pathways exist for glucose metabolism, they all finally lead through the triosephosphate stage to pyruvate. Therefore, in aerated and unaerated cultures, similar end-products of glucose metabolism, which really arise from pyruvate, are detected. The fact that end-products which arise from pyruvate are found in aerated cultures of Esch. coli shows that, whatever the proportion of pyruvate oxidized via the tricarboxylic acid cycle, the cycle definitely does not oxidize all the pyruvate.

Tikka (1935) studied the effect of pH on glucose fermentation. The majority of his results deal with the variation of the end-products arising from glucose with changes in pH. He found that in fermentations by cell suspensions of Esch. coli in alkaline pH values, acetic and formic acids accumulated, while lactic acid accumulated in fermentations at an acid pH. Stokes (1949) confirmed this when phosphate buffer was used in the system, but found that pH had no effect on the end-products of fermentation by a cell suspension of Esch. coli if bicarbonate buffer was used. The pH of the medium must influence the metabolism of glucose during growth. At acid pH values all monocarboxylic acids are produced, while the pH 7.5 cultures produce more acid than can be accounted for by the monocarboxylic acids. This pH must affect the functioning

of enzymes other than phosphoroclastic splitting enzyme and lactic dehydrogenase.



Tikka (1935) reported glucose utilization in two fermentations, at pH 6.3 and 7.6 respectively, in a system containing glucose 6 g., $CaCO_3$ 15 g. and 300 ml. cell suspension of Esch. coli in a total volume of 600 ml.

	Glucose concentration (mg./ml.)	
	pH 6.3	pH 7.6
0	10.0	10.0
1.5	2.8	3.2
3.0	1.3	0.8
5.0	0	0
24.0	0	0

There appears to be very little effect of pH on glucose utilization in Tikka's experiments. Stokes (1949) in his study of the effect of pH on the quantities of metabolic products formed in the fermentation of glucose by Esch. coli, carried out his fermentations in Warburg

manometer flasks and allowed them to continue until all the glucose had been utilized, which he reported usually required 2 to 3 hours. He gave no indication that the rate of glucose fermentation varied with pH. The utilization of glucose by cell suspensions of Esch. coli is definitely dependent on the pH of the system (Fig.3). In stationary phase cultures of Esch. coli the continued utilization of glucose is dependent on the pH of the medium (Tables 4 and 5). The presence of metabolites in the medium, which might be toxic to the cells, does not appear to have any effect on the glucose utilization of unaerated cultures since their removal did not permit an increase in glucose utilization (Table 5).

Dagley et al. (1951c) found that if unaerated cultures were aerated in their stationary phase there was an immediate production of pyruvate. In cultures of A. aerogenes this was followed by an increase in population. There was no growth in cultures of Esch. coli, since Esch. coli is very sensitive to aeration. Lwoff and Monod (1947) reported prolonged lag periods for Esch. coli in aerated media. Dagley et al. (1951c) have shown that pyruvate production was entirely dependent on the presence of glucose and suggested that, in these unaerated cultures, growth ceased due to the failure of reduced DPN to be re-oxidized. This would mean that glycolysis could not

proceed beyond the triosephosphate stage. Ingley et al. (1953), having found that the effect of acidity was more pronounced with unaerated than with aerated cultures, suggested that perhaps hydrogen transfer from reduced DPN to metabolites is inhibited at pH values which permit hydrogen transfer to oxygen.

The findings of this work demonstrate that glucose utilization in stationary phase cultures depends on the initial pH of the medium and not on the degree of aeration. Cultures of initial pH 5.8 are completely unable to utilize glucose in their stationary phase. This is quite compatible with the idea that growth in the unaerated culture in the presence of excess substrates may cease due to the inability of the cells to reoxidize the reduced DPN, thus causing a cessation of glycolysis. Glucose metabolism has ceased also in the aerated culture at pH 5.8. Experiments with washed cell suspensions of Esch. coli showed that glucose metabolism at the final pH of such cultures is almost negligible. In the unaerated culture, then, pH and lack of oxygen to reoxidize reduced DPN are effective in halting growth through preventing glycolysis. The pH of the medium also affects the aerated culture, and glycolysis is stopped here too. The site of action of the pH effect on glucose metabolism must be different in this case for, if this pH was affecting the transfer of hydrogen

from reduced DPN to oxygen, resumption of glycolysis on aeration of unaerated stationary phase cultures would not be obtained.

If growth had ceased in the unaerated cultures at pH 6.5 and pH 7.5 due to the complete conversion of DPN to its reduced form, one would not expect any glucose utilization in the stationary phase cultures. There is a small glucose utilization by stationary phase cultures of initial pH 6.5, while there is quite a reasonable utilization with pH 7.5 cultures. Thus it would appear that a different reason exists for the cessation of growth in these cultures. It is not exhaustion of an essential nutrient since all substrates are provided in excess of requirements. In the unaerated culture at pH 6.5, replacing the cells in fresh media of the same pH and same glucose concentration as the supernatant from the stationary phase culture, i.e., removal of toxic products, did not increase the glucose utilization or cause an increase in population. Hagley et al. (1953) have shown that the final pH cannot be the sole factor causing cessation of growth. It is not the cessation of glycolysis due to lack of DPN. It might be the combined effect of some or all of these factors, but the absolute reasons for growth cessation cannot as yet be defined.

PART II.

INTRODUCTION

The History of Fermentation.

The origin and nature of alcoholic fermentation, the process which Harden (1932) has delightfully termed the conversion of "the insipid juice of the grape into stimulating wine", seems to have irresistibly fascinated natural philosophers from the very earliest times. No reliable estimate can be made of the first observation of fermentation. The earliest chemical literature makes it abundantly clear that the scientists of those times were familiar with the process and, indeed, invoked it to illustrate other mysteries which comprised the 'science' of their day. Historical writings reveal the fact that man has been in possession of alcoholic liquors from time immemorial. Many ideas of the alchemists were based on the phenomenon of fermentation. The subtle change in the properties of the fermenting liquid, the vigorous frothing and the resulting product all seemed to offer an illustration of the mysterious and long-sought for transformation of the base metals into gold, mediated by the elusive 'philosopher's stone'. As chemical science emerged from the mists of alchemy, more definite knowledge of alcoholic fermentation was obtained. In the time of the phlogistic chemists a theory of fermentation was proposed, firstly by Willis (1659) and later by Stahl (1697). Their fundamental

proposal, which formed the basis of many subsequent theories, was that a violent internal motion of the fermenting substance was readily communicated to the aqueous liquid surrounding it. As a result, the combination of the constituents of the liquid was disturbed and new particles were formed. Thus a fermenting liquid on addition to an aqueous solution can cause this liquid to ferment also. By the time of Lavoisier, this theory was generally accepted and alcoholic fermentation was known to require the presence of sugar and was thought to lead to the production of carbon dioxide, acetic acid and alcohol.

Lavoisier, who established the composition of organic compounds as carbon, hydrogen and oxygen, systematically analysed the substances concerned in fermentation. He concluded (1789) that the sugar was split into two parts, one of which was oxidized at the expense of the other to form carbonic acid, whilst the other was reduced to alcohol. Such a conclusion in the light of later discoveries was very nearly accurate, but is clearly one of those remarkable instances in which the genius of the investigator triumphs over experimental deficiencies, since Lavoisier's analytical figures were far from accurate.

It had been found that food could be preserved by placing it in sealed bottles and heating these in boiling water. Gay-Lussac (1810) observed that when such bottles

were opened to the air fermentation occurred. Since heating always stopped the fermentation, while the admission of fresh oxygen always caused its recommencement, he concluded that the fermentation was effected by some substance formed by the action of oxygen on the liquid, and that this substance was unstable to heat. There seems to have been no suspicion in the scientific minds of this time that fermentation was anything else but a purely chemical reaction. Even after the microscopic study of yeast, this was still regarded as a chemical compound.

Then no less than three observers (Cagniard-Latour, 1838, Schwann, 1837, and Kutzing, 1837) almost simultaneously hit upon the secret of fermentation and declared that yeast was a living organism. They found that fermentation only occurred in the presence of living yeast cells, which propagated themselves in the fermenting liquid. These three observations were received with incredulity and then reviewed with scorn by Berzelius, who regarded yeast as a chemical catalyst. To the scorn of Berzelius was soon added the sarcasm of Wohler and Liebig (1839). A paper by Turpin (1838) supporting the three "heretics" provoked Wohler into writing an elaborate skit on the subject, to which Liebig added some of his own touches, and then had published in 'Annalen' following immediately after a translation of Turpin's paper. In it, yeast was described with

a considerable degree of anatomical realism as consisting of eggs which developed into minute animals, shaped like a distilling apparatus, by which the sugar was taken in as food and digested into carbonic acid and alcohol, which were separately excreted, the whole process being easily followed under the microscope.

Gradually a strong body of evidence was accumulated in favour of the vegetable form of yeast, and soon even Berzelius supported this view. At the middle of the nineteenth century opinions regarding alcoholic fermentation were still divided. On the one hand, Liebig's theory that fermentation was a purely chemical reaction and was brought about by the transmission of instability from an unstable and changing ferment, causing a decomposition of the sugar, was widely held and taught. On the other hand, it was held that accompanying alcoholic fermentation there was a development of a living organism, the yeast, and that fermentation was a phenomenon due to the life and vegetation of this organism. In 1857, Pasteur began his classical researches. He expressed his conclusions thus: "The chemical act of fermentation is essentially a phenomenon correlative with a vital act, commencing and ceasing with the latter. I am of opinion that alcoholic fermentation never occurs without simultaneous organisation, development, multiplication of cells, or the continued

life of cells already formed". Liebig, however, would not accept these conclusions, and it was not until 1872, after a long controversy between the two 'masters', that Pasteur's ideas were fully accepted, and it became generally recognised that fermentation changes were due to specific organisms, which produced them in the exercise of their vital functions.

This was, however, not the end of the story. Traube (1858) had enunciated a theory that all fermentations produced by living organisms were caused by ferments, which are definite chemical substances produced in the cells of the organism. These ferments were closely linked with proteins, and it was their function to transfer oxygen and hydrogen of water to different parts of the molecule of the fermentable substance and thus bring about the apparent intramolecular oxidation and reduction which is so characteristic of fermentation changes. This theory attracted much attention and for many years, numerous and repeated experiments were undertaken to find evidence for it. Despite Pasteur's reiterated assertion that all fermentation phenomena were manifestations of the life of the organism, and the numerous unreliable and negative results obtained by such experiments, the search went on. Then, Buchner (1897) obtained a liquid from yeast which, in the

complete absence of cells, fermented sugar into carbon dioxide and alcohol.

This discovery introduced a new method for attacking the problem of alcoholic fermentation. This is not a process specific to yeast, but is also a function of muscle, bacteria, moulds and higher plants in their decomposition of carbohydrates, although these fermentations do not all result in the production of alcohol. When this work was undertaken in 1952 the mechanism of alcohol production in bacteria had never been fully elucidated. It seemed therefore that a study of the production of ethanol by Escherichia coli would be a useful contribution to our knowledge of bacterial metabolism.

Glucose Fermentation.

Harden (1901) showed that Bacillus coli communis fermented glucose according to the equation:

Glucose ⟶ Lactic + Acetic + Ethanol + Succinic, CO_2, H_2.

Moles: 1 1 0.5 0.5 (traces)

The molecular ratio of acetic to ethanol remained fairly constant for various coliform organisms and usually approximated to 1. Harden deduced that ethanol arises from the grouping $CH_2OH.CHOH-$, since mannitol with two such groupings gives twice as much ethanol as glucose, which has only one. This hypothesis was further supported by the finding that glycerol, which has one of these groups, yields similar amount of ethanol as glucose, on the mole to mole basis.

The occurrence of acetic acid and ethanol in a 1:1 ratio led Harden to postulate a common precursor of these compounds. Analogy with yeast fermentations led later workers to presume this to be acetaldehyde. Rosbytchor (1912) first suggested that acetaldehyde was an intermediate in alcohol fermentation. Grey (1913) showed, by the colour produced by the fermentation mixture with Schiff's reagent, that small quantities of acetaldehyde did occur during glucose fermentation. As the ratio of acetaldehyde to ethanol was constant, he decided that the production of aldehyde was related to the formation of

ethanol, CO_2 and acetic acid rather than to the other fermentation products. Grey compared a normal strain of B. coli communis with an artificially selected strain which had lost the ability to produce gas from glucose, and from the ratio of his results, he concluded (1914) that ethanol and acetic acid arise from a common precursor which bears a constant ratio to the formic acid produced. He suggested that this intermediate substance was probably acetaldehyde. Schaffer (1920) studied the fermentation of glucose by the coliform organisms, and postulated that pyruvate gave rise to acetaldehyde, which was then reduced to ethanol. A general property of aldehydes is that they undergo the Cannizaro reaction, which is the oxidation of one molecule of aldehyde at the expense of the other, which is reduced. Thus from acetaldehyde, ethanol would arise by reduction, acetic acid by oxidation.

Grey (1919) showed that the fermentation mechanisms of mannitol and glucose were similar. Kay (1926) worked along similar lines to Harden and compared the fermentations of mannitol, glucose, gluconic acid, glycuronic acid and saccharic acid. He concluded that as they all gave rise to the same products, they were all being fermented in the same way. A comparison of the product ratios however showed that more reduced products arose from the reduced sugars whereas more oxidized products arose from the oxidized substrates. Johnston, Peterson and Fred

(1931) using acetone-butyl alcohol fermentation organisms studied the fermentation of glucose, mannitol and gluconate. They also found that the more reduced substrates gave rise to a greater proportion of reduced end-products, and the more oxidized substrates gave more oxidized end-products.

Virtanen, Karstrom and Turpeinen (1929) studied the fermentation of glucose and dihydroxyacetone by B. coli. Glucose gave lactic acid (40%), succinic acid (2%), acetic acid (10%), ethanol (10%) and a little formic acid. Dihydroxyacetone yielded glycerol (42.6%), acetic acid (30%) and formic acid (17%). They suggested that the first step in the dihydroxyacetone fermentation was a dismutation of two triose molecules through glyceraldehyde, and to support this showed that glyceric acid gave acetic and formic acids. L-glyceric acid was shown by Virtanen and Peltola (1930), however, to give rise to not only acetic and formic acids but also to ethanol, while Antoniani (1933b) gave as the products of the fermentation of DL-glyceric acid, acetic and formic acids, very little ethanol and no lactic acid. Virtanen and Hausen (1932) found that L-glyceraldehyde yielded glycerol, acetic, formic and lactic acids. Both fresh cells and dry preparations of Esch. coli were found by Antoniani (1933a) to give pyruvic acid (73%) with a little

ethanol from phosphoglyceric acid. Tikka (1935) carried out fermentations with washed cell suspensions of Esch. coli at different pH values. He found that whereas the amount of ethanol produced remained constant at different pH values, lactic acid accumulated at acid values (pH 6.4 and below), while acetic acid and formic acid predominated at alkaline values (pH 7.4 and 7.6). The fermentation of phosphoglycerol gave ethanol, of phosphoglyceric acid acetic acid, with formic acid presumably as the other product in each case. In the presence of toluene, phosphoglyceric acid yielded pyruvic acid, which in turn yielded acetic, formic and lactic acids. From these findings Tikka originated a scheme for glucose fermentation and ethanol production which has been repeated in text books such as that of Gale (1951). This scheme has been superseded by the Embden Meyerhof Parnas one, the primary mechanism of which results in the production of pyruvate. All the reactions of this scheme except one have been studied and established in Esch. coli. We have previously discussed the oxidative pathway of glucose utilization, and the E.M.P. scheme in more detail.

Pyruvate Metabolism.

In muscle the pyruvate is reduced to lactic acid, with the regeneration of the cozymase required for the oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid. In yeast, the pyruvic acid is attacked by the enzyme carboxylase to form acetaldehyde which also acts as a hydrogen acceptor for reduced cozymase and is reduced to ethanol. In general, a carboxylase enzyme has not been demonstrated in bacteria. Although they can oxidatively decarboxylate pyruvate, there is very little evidence in favour of an anaerobic decarboxylation of the carboxylase type. Anaerobically acetic, lactic, formic, succinic, ethanol and CO_2 are formed from pyruvate (Wilks, 1936). Still (1941) found that Esch. coli oxidized pyruvate to CO_2 and H_2O. Cell free extracts were prepared which were capable of oxidizing and dissimilating pyruvate. There was a very slow oxidation of acetaldehyde, and this could not be detected during pyruvate metabolism. The system required phosphate, cocarboxylase (diphosphothiamin or DPT) and Mn^{++} (or Mg^{++}). Yeast under similar treatment would yield a cell-free extract containing carboxylase, but no carboxylase was detected in the extract of Esch. coli. Kandalis (1951) reported during studies on the effect of dihydrostreptomycin on the anaerobic dissimilation of

pyruvate by Esch. coli, that the normal products of this dissimilation were acetic and formic acids (75%) and a decarboxylated non-acidic end product (25%). This latter product was not identified. Sadana (1954) obtained ethanol, acetyl phosphate and CO_2 from the oxidation of pyruvate by Desulphovibrio desulphuricans. Acetaldehyde was not utilized as a substrate. On the other hand, King and Cheldelin (1954) have found a pyruvic carboxylase in cell-free extracts of Acetobacter suboxydans. The first step in pyruvate oxidation was decarboxylation, yielding free acetaldehyde, followed by oxidation. The carboxylase was separated from the oxidative enzymes and shown to require DPT and Mg^{++}.

Different species ferment the pyruvate to different end-products, and even in the same species the fermentation of pyruvate can differ with environmental conditions. This gives rise to the complexity of bacterial fermentations, which can be classified by their major end-products.

Homolactic and Heterolactic Fermentations.

The homolactic type of fermentation accounts for more than 90% of the glucose as lactic acid. The hetero lactic fermentation yields 50% lactic acid, with the rest of the pyruvate forming ethanol, acetic acid, CO_2 and some glycerol.

Propionic Fermentation.

The propionic fermentation results in propionic, acetic and succinic acids. Under certain conditions, lactic acid can be produced. Wood, Stone and Workman (1937) proposed a scheme for the intermediary metabolism of propionic acid bacteria in which acetic acid gives rise to succinic acid which is then decarboxylated to propionic acid. It was first observed by Wood and Workman (1938) that in a propionic fermentation of glycerol carried out in a closed system in an atmosphere of CO_2 the total carbon of the end products exceeded that of the carbohydrate fermented. This they attributed to fixation of CO_2 in the fermentation products. Furthermore they were able to show that at each stage in the fermentation the moles of CO_2 fixed and succinic acid produced were approximately equal. Wood, Workman, Hemingway and Nier (1941a) allowed a cell suspension of Propionibacterium to ferment glycerol and glucose in the presence of $NaHCO_3$ enriched with ^{13}C. This ^{13}C appeared in the succinic acid, propionic acid and propyl alcohol. The succinic acid was degraded by Wood, Workman, Hemingway and Nier (1941b) and their findings agreed with the proposal that the succinic acid arose by the union of a C_3 and C_1 compound. Krebs and Eggleston (1941) showed that oxaloacetate could be

converted to succinic acid. Werkman and Wood (1942), Kelwicke (1948) and Johns (1951) have all shown that succinic acid could be decarboxylated to propionic acid. Thus we have the scheme:

Glucose
↓
Pyruvate $\xrightarrow{+ CO_2}$ Oxaloacetate ⟶ Succinate ⟶ Propionate $+ CO_2$

An enzyme capable of fixing CO_2 in pyruvate has been obtained from Micrococcus lysodeikticus by Krampitz and Werkman (1941) and from Esch. coli by Kalnitsky and Werkman (1943).

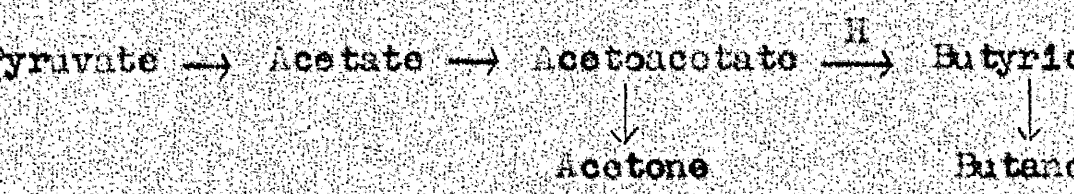
Other findings do not support this scheme. Wood and Werkman (1940) pointed out that although the Embden Meyerhof scheme is fluoride sensitive, propionic acid fermentation is not and therefore there must be a second mechanism of fermentation. Wood and Leaver (1953), studying the CO_2 turnover in fermentations by the propionic acid bacteria, concluded that the succinate is not decarboxylated to CO_2 and propionate but is split into propionate and some C_1 compound, other than CO_2. Leaver and Wood (1953), reviewing the subject, present evidence from the fermentation of labelled substrates which is inconsistent with these previous concepts of the propionic acid fermentation. Firstly they found that not only is the Embden Meyerhof fermentation not the sole one, but that the

proposed hexose monophosphate shunt mechanism, although functioning, cannot be the only other mechanism. There must be yet another pathway for propionic acid fermentation. They turned to the mechanism which Gibbs and De Moss (1951) found in Leuconostoc mesenteroides, but this did not function. They showed that the C_1 compound postulated by Wood and Leaver (1953) could be formaldehyde, which was produced and utilized during the fermentation of glycerol by Propionibacterium arabinosum. They also were led to the conclusion that all the propionic acid could not arise from succinic, but failed to find any other precursors.

Butyric and Butyl Fermentations.

The butyric acid fermentation results in the formation of acetic acid, butyric acid, CO_2 and H_2. In the butyl fermentation, these products are accompanied by ethanol, butanol and acetone. In yet another group of organisms, isopropanol is also produced. The formation of the solvents is dependent on the pH of the reaction. Osburn, Brown and Werkman (1937) found that the normal end products of the fermentation of glucose by Clostridium butylicum Donker are butyl and isopropyl alcohols, CO_2, H_2, with small quantities of acetic and butyric acids, when the cells were grown at pH 6.0. When the pH was raised

to 7.0 by the addition of $NaHCO_3$, the formation of the alcohols was suppressed, while formic, acetic, butyric, lactic and pyruvic acids accumulated. Davies and Stephenson (1941) studied the products during the growth of Clostridium acetobutylicum and found that when the pH fell below 6.0 the fermentation changed from a butyric one to a butylic one, and the acids were reduced to the corresponding alcohols. The mechanism of this fermentation has been studied isotopically by Wood, Brown and Werkman (1945). They found that carboxyl labelled acetic acid gave rise to butanol labelled in the C_2 and C_4 positions, and acetone and isopropanol labelled in the C_2 position. C_2, C_4 labelled butyric acid gave rise to similarly labelled butanol, containing 85% of the added isotope. Labelled acetone gave rise to labelled isopropanol. It was found by Rosenfield and Simon (1950a) that the yield of acetone was increased by more than 100% if K^+ and Mg^{++} were added. Mg^{++} is a cofactor for acetoacetate decarboxylase and the most obvious mechanism would seem to be:



Johnston, Peterson and Fred (1933) found that Cl. acetobutylicum grown under conditions suitable for the production of acetone rapidly decarboxylates aceto-acetate. Acetoacetate decarboxylase has been isolated from acetone treated cells. Thus it seems definite that acetone arises from aceto acetate. Davies (1942) failed to show that acetoacetic acid could be reduced to butyric acid. Rosenfield and Simon (1950b) observed that phosphoenol pyruvate gave rise to the same end products as pyruvate and they suggested the scheme proposed above is reversed in the production of butyric acid from acetate.

Acetate → Pyruvate → Phosphoenol Pyruvate → Unknown Condensation Product.

This, however, does not agree with the isotopic results found by Wood et al. (1945).

The Coli-Aerogenes Fermentation.

The coli-aerogenes fermentation is a very heterogeneous one. This group of organisms produces lactic, acetic, formic, succinic acids, ethanol, CO_2 and H_2 with in some cases acetoin and 2,3 butylene glycol. The pyruvate is converted to lactic acid by the reversal of the lactic dehydrogenase system, or by dismutation with the formation of acetic acid, lactic acid and CO_2. The succinic acid may arise by the fixation of CO_2 in pyruvate, as in the propionic acid fermentation, or by the condensation of two C_2 molecules. Harden (1901) postulated that succinic acid was formed by the condensation of two molecules of acetic acid, and this was supported by Stone, Wood and Werkman (1936) and Stone, Wood and Werkman (1937). Using washed cell suspensions of Aerobacter aerogenes, $CH_3{}^{13}COOH$ was added to the fermentation of glucose. Significant amounts of succinic acid with ^{13}C in the carboxyl group were isolated. The addition of succinate containing carboxyl labelled with ^{13}C resulted in the isolation of $CH_3{}^{13}COOH$. This could not arise through the intermediates fumaric, malic, oxaloacetic, pyruvate, acetic, because the acetic in this case would be unlabelled. Thus it was envisaged that two molecules of acetic could condense to succinic acid, and that this in turn could be split into two acetate molecules. The CO_2 and H_2 of

Mechanisms of Acetoin Production from Ochoa (1951).

Scheme 1 :

Pyruvate + Enzyme ⟶ (Acetaldehyde - Enzyme) + CO_2

↓ + Pyruvic Acid

Acetoin + CO_2 ⟵ Acetolactic Acid + Enzyme.

Scheme 2 :

Pyruvate + Enzyme ⟶ (Acetaldehyde - Enzyme) + CO_2

↓

Acetaldehyde + Enzyme

Pyruvate + Enzyme ⟶ (Acetaldehyde - Enzyme) + CO_2

↓ + Acetaldehyde

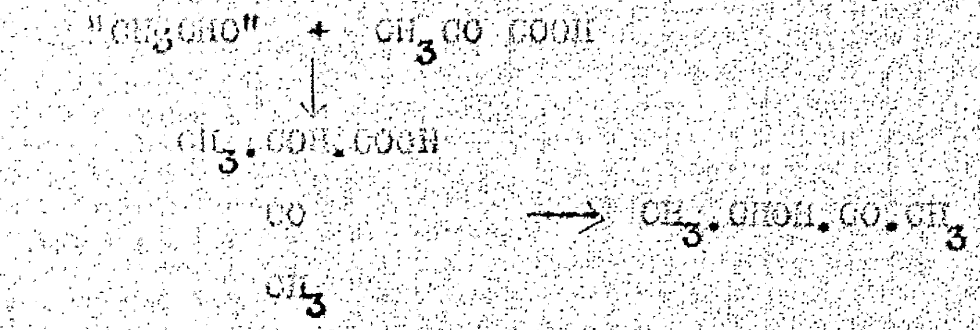
Acetoin + Enzyme

these fermentations arise from formic acid by the action of formic hydrogenlyase, a reaction which was studied as early as 1901 by Pakes and Jollyman. This enzyme is adaptive. It is almost completely absent from aerobically grown cultures, while present in considerable concentration in those grown anaerobically.

Acetoin Formation.

There appear to be two mechanisms for the formation of acetoin. Ochoa (1951) explained these by the reactions shown opposite. In bacteria, which normally produce acetoin we have scheme 1, while scheme 2 was postulated to account for the condensation of added acetaldehyde by the system from yeast and animal tissues, where α-acetolactic acid is not an intermediate. Silverman and Werkman (1941) obtained a cell-free extract from A. aerogenes which formed acetoin from pyruvic acid. Diphosphothiamin and Mg^{++} or Mn^{++} were required as co-factors. Crude cell-free extracts from bacteria which would produce acetoin from pyruvate were found to decarboxylate α-acetolactic acid (Watt and Krampitz 1947, Krampitz 1948, Juni 1950). Juni (1952) confirmed this and showed that it was a specific decarboxylation, and that the enzyme was present in bacteria which normally produce acetoin in their fermentations. He resolved the bacterial extract

Mechanism of Acetoin Production proposed by Juni (1952).



Mechanism of Acetoin Production postulated by Happold and Spencer (1952).

Pyruvate + Enzyme ⟶ (Acetaldehyde - Enzyme) + CO_2

Pyruvate ⟶ Acetate + CO_2

(Acetaldehyde - Enzyme) + Acetate ⟶ Diacetyl + Enzyme

Acetoin

Production of Acetoin by Escherichia coli Extract from Acetyl Phosphate (Chin and Gunsalus, 1954).

$CH_3COOPO_3^=$ + HS.CoA ⟷ $CH_3.CO - S\ CoA + HPO_4^=$

$CH_3CO - S\ CoA + CH_3CHO + LIP\ (SH)_2$ ⟶

$CH_3CHOH.CO.CH_3 + LIP\ (S)_2 + CoA\ SH.$

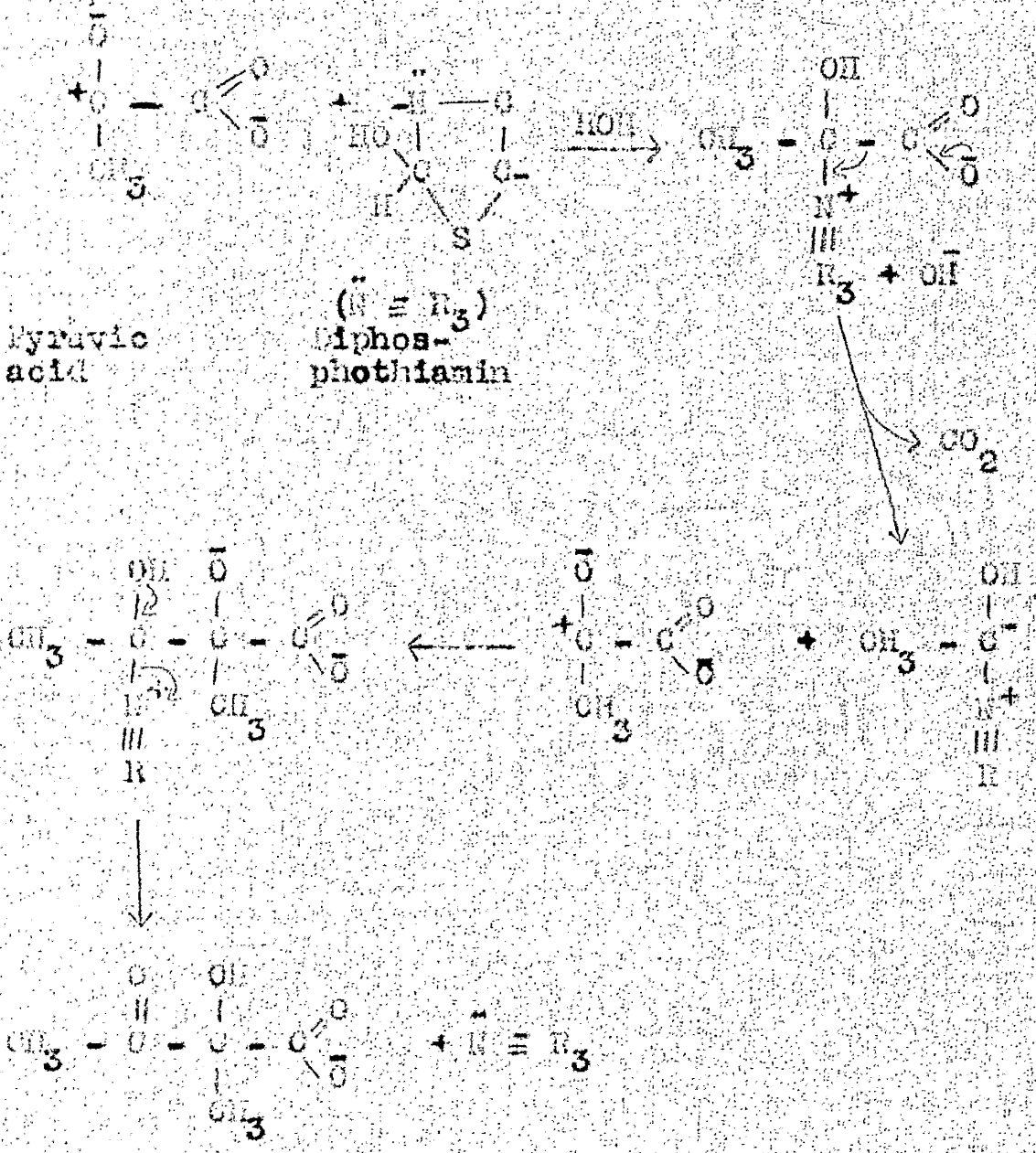
CoA SH = Reduced coenzyme A.

$LIP(SH)_2$ = Reduced lipoic acid.

$LIP(S)_2$ = Lipoic acid.

into two components. The first of these forms α-acetolactic acid from pyruvate, and is diphosphothiamin dependent, the second decarboxylates this to acetoin. He ruled out the possibility that acetaldehyde is an intermediary, and suggests that some "active acetyl" compound at the same oxidation level as acetaldehyde combines with pyruvic acid to form the α-acetolactic acid. This is described in the scheme opposite. Work by Happold and Spencer (1952) supported Juni's findings for the production of acetoin by A. aerogenes, but also gave evidence that another system was present in these cells. This is similar to the one found in yeast and animal tissues. α-Acetolactic acid is not an intermediate, and acetate was condensed during the decarboxylation of pyruvate. Happold and Spencer (1952), therefore, postulate the reactions in the scheme shown opposite. Chin and Gunsalus (1954) obtained an extract from Esch. coli, an organism which does not have acetoin as a normal end-product, which would catalyze the formation of acetoin from acetyl phosphate in the presence of phosphotransacetylase, reduced lipoic acid ($Lip(SH)_2$), Coenzyme A (CoA), DPT, Mg^{++} and acetaldehyde. Their proposed pathway is also shown opposite. Kobayashi and Kalnitsky (1954) investigated the formation of α-acetolactic acid from pyruvate by cell-free extracts of Proteus morganii and they suggested the reaction

The Mechanism of α-acetolactate Formation (Kobayashi and Kalnitsky, 1954).



α-acetolactic acid

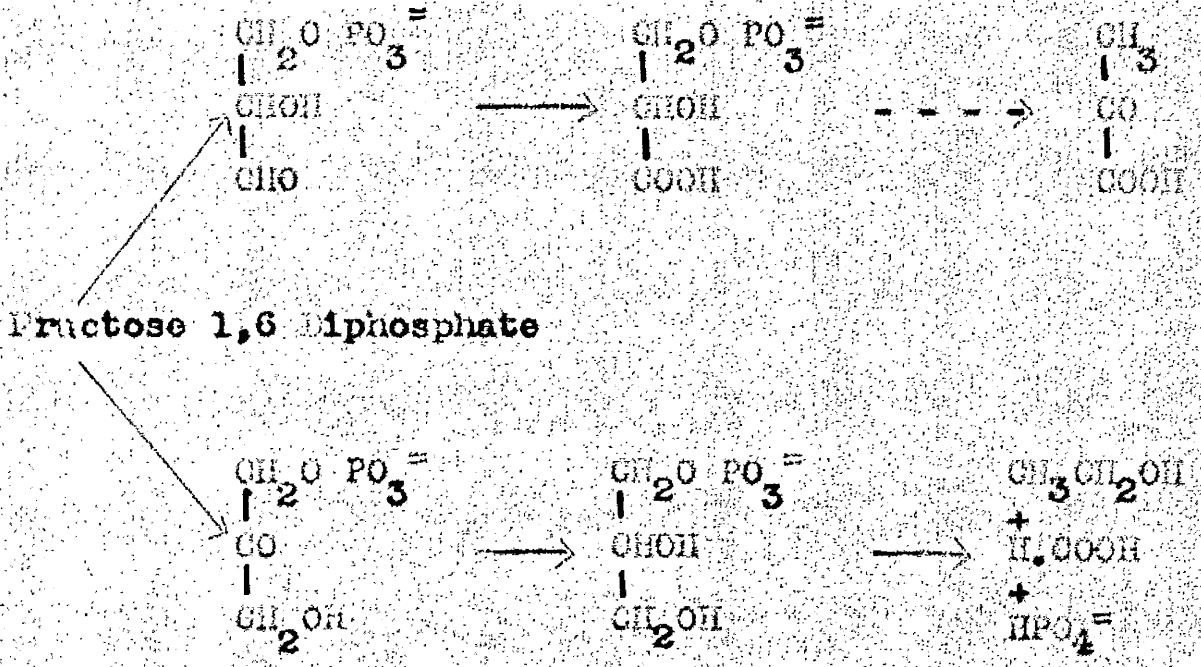
sequence shown opposite to account for the role of DPN.

In these fermentations, the ratio of the end-products depends on the environment. In Esch. coli for example, more acetic and formic acids occur at an alkaline pH, while lactic acid accumulates at acid pH values. In A. aerogenes, although the enzymes for both reactions are present, at pH 8.0 only acetic and formic acids are formed, while below pH 6.9 the pyruvate forms acetoin only. The formation of acetic and formic acids from pyruvate will be discussed later.

Fermentation of Glycerol.

With the elucidation of the Embden Meyerhof scheme, it became known that by the action of aldolase the triose-phosphates, phosphoglyceraldehyde and phosphodihydroxy acetone are formed. The oxidation of phosphoglyceralde-hyde to phosphoglyceric acid forms half of an oxido-reduction reaction, the other half of which is the re-duction of phosphodihydroxyacetone to α-glycerophosphate. According to Tikka (1935) this α-glycerophosphate then gives rise to ethanol, formic acid and phosphate. Dale (1951) points out that since phosphoglyceraldehyde and phosphodihydroxyacetone are in equilibrium, and since the former gives rise to pyruvic acid and the latter ethanol, the proportions of pyruvic acid to ethanol formed will be

Proposed Scheme for Ethanol Production by Gale (1951).



dependent on the reaction velocities of the various intermediate reactions involved. Furthermore, the oxidation of phosphoglyceraldehyde can be coupled to the reduction of either phosphodihydroxyacetone or pyruvic acid. Since pyruvic acid cannot accumulate until the glycolytic cycle has proceeded through all the intermediate stages, reduction of phosphodihydroxyacetone will predominate during the starting up of the cycle. Gale has summarized this in the scheme facing this page. He gives no indication of the mechanism which produces the ethanol from the α-glycerophosphate. Obviously as the α-glycerophosphate is formed by the reduction of phosphodihydroxyacetone, the further metabolism of the α-glycerophosphate to ethanol must be by some other pathway than the Embden Meyerhof one, as this would necessitate the oxidation of α-glycerophosphate back to phosphodihydroxyacetone. However, Underkofler and Palmer (1937) showed that the general reaction of Acetobacter bacteria on glycerol was the production of dihydroxyacetone. Mickelson and Shideman (1947) found that glycerol and α-glycerophosphate were oxidized rapidly by Escherichia freundii. The rate of oxidation of glycerol was increased by the addition of adenosine triphosphate (ATP) and inorganic phosphate, and they suggested that the pathway for glycerol oxidation is via α-glycerophosphate to the triosephosphate

stage and thence by the Embden Meyerhof route. Gunsalus and Umbreit (1945) studied the products of anaerobic glycerol fermentation by Streptococcus faecalis and concluded that the fermentation followed the normal glucose pathway. They found that anaerobic fermentation of glycerol was dependent upon the presence of hydrogen acceptors in the media. Thus we have:

Glycerol ⇌ Glycerophosphate ⇌ Phosphodihydroxyacetone

↓

Pyruvic Acid.

It seems most unlikely therefore that ethanol is produced by some unknown mechanism from α-glycerophosphate, since this compound is metabolized through the Embden Meyerhof scheme, and even the more recent finding of another possible pathway still leaves no doubt but that ethanol in fermentations arises only from pyruvic acid.

Asnis and Brodie (1953) isolated from Esch. coli a DPN-specific glycerol dehydrogenase, which catalyzed the oxidation of glycerol to dihydroxyacetone. They found that phosphate did not participate in the reaction. The metabolic pathways of glycerol dissimilation were studied by Magasanik, Brooke and Karibian (1953) with two strains of A. aerogenes. Strain 1033 grew aerobically and anaerobically on glycerol, and cell suspensions fermented

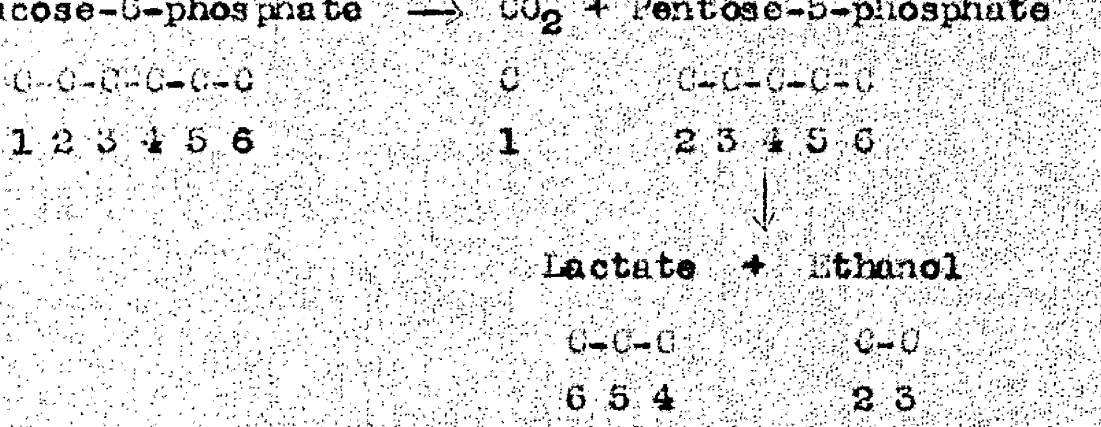
glycerol in the presence of a hydrogen acceptor. They postulated a scheme, with a glycerol dehydrogenase giving dihydroxyacetone, which is then converted via glyceraldehyde to pyruvic acid. The reversibility of the glycerol-dihydroxyacetone reaction was shown, and has previously been observed in Esch. coli grown on dihydroxyacetone (Virtanen et al. 1950). As glycerol accumulated during the fermentation of glyceraldehyde, this was assumed to be on the pathway. The conversion of glyceraldehyde to pyruvic acid was not studied. Under aerobic conditions, the pyruvate was oxidised: under anaerobic conditions, it was split to formic and acetic acid: the acetic acid then acted as a hydrogen acceptor and was converted to ethanol. Strain 1041 oxidized only glycerol and produced the same end products as strain 1033 under aerobic conditions. It, however, oxidized the glycerol by a glycerokinase to α-glycerophosphate which then yielded pyruvic acid. The intermediate steps between α-glycerophosphate and pyruvate were not investigated, but it was assumed that it entered the Embden Meyerhof pathway through phosphodihydroxy acetone. Vinas, Bourgeois and Leubion (1954), studying glycerol oxidation by Bacillus subtilis, have postulated that the first product in the oxidation is dihydroxyacetone, which is then phosphorylated by ATP to phosphodihydroxyacetone. This second reaction is known

in animals (Lindberg, 1951). Hauge, King and Cheldelin (1954) have also suggested that the oxidation of dihydroxyacetone by cell suspensions and cell-free extracts of Acetobacter suboxydans involves phosphorylation as the primary step.

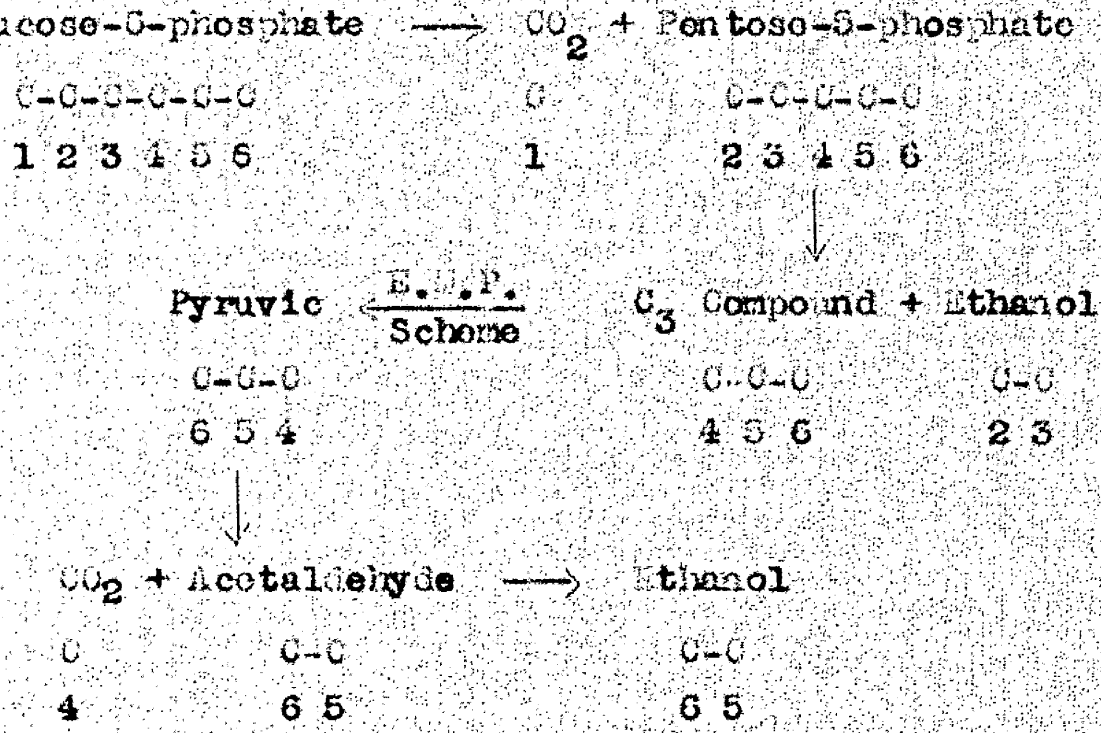
Heterolactic Fermentation.

Gunsalus, De Moss and Bard (1951) and Gunsalus and Gibbs (1952) studied the heterolactic fermentation of Leuconostoc mesenteroides. This organism produces equimolar amounts of lactate, ethanol and CO_2, from hexose, yet does not appear to possess either an aldolase or an isomerase enzyme. The Embden Meyerhof scheme, therefore, cannot operate completely in its metabolism. They succeeded by chemical and isotopic experiments in demonstrating a fission of glucose to CO_2 and a C_5 compound, which was then split to lactic acid and ethanol. The ethanol produced arose from the second and third carbon atoms of glucose. The addition of labelled acetate to the fermentation of unlabelled glucose resulted in the incorporation of the isotope in ethanol, the amount of incorporation being proportional to the concentration of labelled acetate. From the specific activities found and the quantity of isotope appearing in the ethanol, they concluded that the added acetate was not in equilibrium with the acetate formed by the cells during glucose

Mechanism of Ethanol Formation from Glucose by Leuconostoc mesenteroides.



Mechanism of Ethanol Formation from Glucose by Pseudomonas lindneri.



fermentation, and, therefore, acetate is not an obligatory intermediate in the formation of ethanol by this mechanism.

Gibbs and De Moss (1951) investigated ethanol formation in Pseudomonas lindneri. This organism ferments glucose as shown by the equation:

Glucose ⟶ Lactic + Ethanol + CO_2

Moles: 1 0.2 1.8 1.8.

It possesses a carboxylase enzyme, and also phosphorylative systems. The fission of the glucose molecule in Ps. lindneri, studied by isotopic techniques, resembles that found in L. mesenteroides, but the subsequent metabolism is different. The ethanol in Ps. lindneri fermentations arises by two mechanisms as shown opposite, where the mechanisms found in both these organisms are given. The work carried out on these fermentations has been summarized by De Moss (1953).

Acetaldehyde in Metabolism.

Acetaldehyde has been found in small amounts in bacterial fermentations. It is usually considered to be fairly toxic to micro-organisms, if in high concentrations, but there is increasing evidence that it plays an important part in metabolism. Still (1940) showed that Esch. coli possessed an alcohol enzyme which catalysed the oxidation of ethanol to acetaldehyde:-

$$CH_3CH_2OH \rightleftharpoons CH_3CHO + 2H$$

It is not known whether this enzyme plays a part in the production of alcohol by Esch. coli, but it is not unreasonable to assume that it does. Racker (1951) obtained extracts of Esch. coli, which were capable of catalysing the reversible reaction:

Glyceraldehyde Phosphate + Acetaldehyde $\rightleftharpoons$ Desoxypentose Phosphate.

Acid to Alcohol.

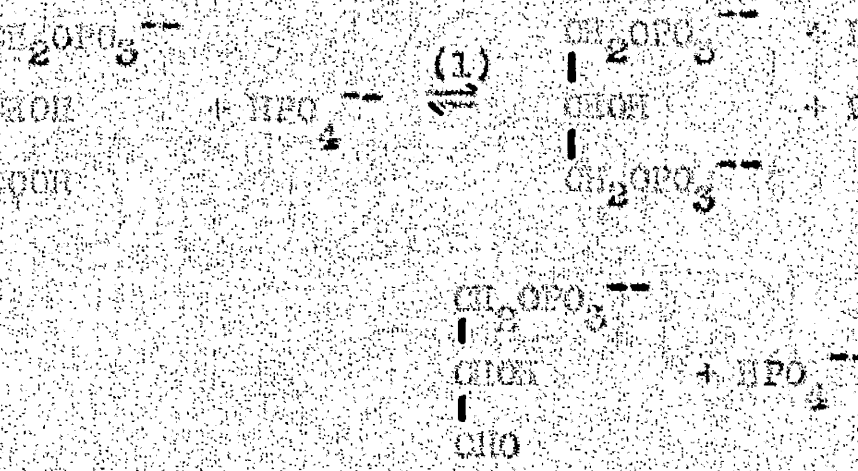
Stokes (1949) studied the fermentation of glucose by cell suspensions of aerobically grown Esch. coli, which therefore contain no formic hydrogenlyase, and concluded that the fermentation could be represented by the equation:

Glucose $\rightarrow$ Lactic + Acetic + Ethanol + Succinic + Formic Acids

Moles: 1 0.2 0.8 0.8 0.4 1.2.

He found that pH affected the yields of metabolic products from fermentations conducted in phosphate buffer, whereas it had no effect on fermentations carried out in bicarbonate buffer. Stokes suggested that the most probable mode of formation of ethanol is by reduction of acetate, but he gave no experimental work in support of this view. Many workers, as Elsden (1952) pointed out, have considered the possibility of reduction of a fatty acid, via the aldehyde, to the corresponding alcohol, but have been deterred from completely accepting this by the fact that such a reduction is a matter of considerable difficulty

in chemical laboratories. Elsden called this an *in vitro* attitude, since Warburg and Christian (1939) in their study of 3-phosphoglyceraldehyde dehydrogenase, have demonstrated a biological mechanism for the reversible reduction of a carboxyl group to a carbonyl group. The key process is the formation of an acyl phosphate from the carboxylic acid, which then becomes readily reducible. Thus 3-phosphoglyceric acid is firstly converted to 1,3-diphosphoglyceric acid, which can then be reduced to 3-phosphoglyceraldehyde.

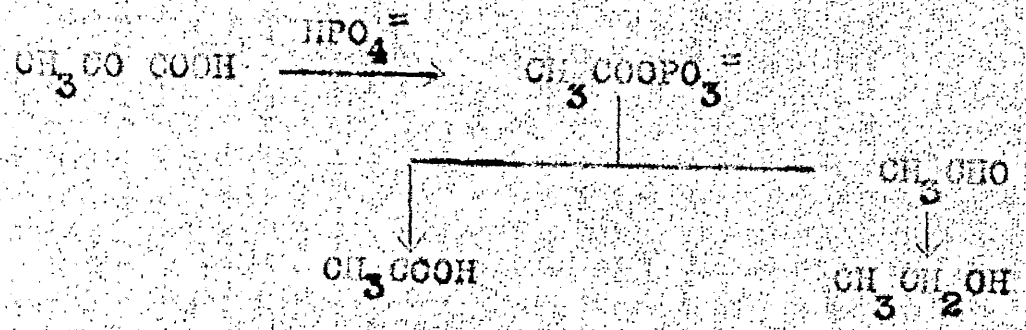


The reverse of reaction (2), the formation of 1,3-diphosphoglyceric acid from 3-phosphoglyceraldehyde, has now been demonstrated to occur in two stages. Cosper (1954) has shown that 3-phosphoglyceraldehyde is oxidized first to a 3-phosphoglyceryl-enzyme complex, which is then phosphorylated to the diphosphoric acid. If reactions (1) and (2) represented the general mechanism for the conversion of a carboxylic acid to the corresponding aldehyde,

it was to be expected that the reduction of acetic acid to acetaldehyde proceeds via acetyl phosphate. Stadtman and Barker (1949) showed that the oxidation of acetaldehyde to acetic acid by extracts of Clostridium kluyveri required inorganic phosphate and they detected acetyl phosphate as a product of the reaction. This resembled the oxidation of 3-phosphoglyceraldehyde, but so far the reaction has not been reversed and has been demonstrated in only one organism.

Theoretical considerations prevented unreserved acceptance of the idea of direct reduction of a fatty acid to an alcohol, but many observations made in the course of experimental work were difficult to explain in any other terms. Thus Spenkman (1920) showed that the addition of butyric acid to a butanol fermentation led to an increase in the amount of n-butanol formed, and added propionic acid was converted to n-propanol, a substance not normally produced in this fermentation. Peterson and Fred (1932) and Davies and Stephenson (1941) analysed the medium at different times during the growth of Cl. acetobutylicum and found that the acids which accumulated in the early stages were converted to the corresponding alcohols when the pH fell below 5.0 in the latter stages of the experiments. Davies (1942) again showed that washed suspensions of Cl. acetobutylicum which were fermenting glucose,

echanism for Ethanol Formation proposed by Elsden (1952).



converted added butyric acid to n-butanol. Mickelson and Werkman (1939) found that propionic acid and propionaldehyde were reduced to n-propanol by Aerobacter indologenes fermenting glucose.

Final proof of the conversion of fatty acids to their corresponding alcohols had to await the preparation of labelled fatty acids. The studies of Slade and Werkman (1943) on the metabolism of labelled acetate by Aerobacter indologenes fermenting glucose showed that the acetate is converted to succinic acid and also to ethanol. Wood et al. (1945) showed that Cl. butyricum and Cl. acetobutylicum produced a small amount of ethanol from acetate. They also found that if carboxyl-labelled butyric acid was added to the fermenting mash, it was reduced to n-butanol. On the basis of this data, Elsden (1952) concluded that there could be little doubt as to the mechanism employed in the formation of alcohols. Ethanol, in particular, would be produced by the reduction of acetate - probably in the form of acetyl phosphate - to acetaldehyde, which would then be further reduced to ethanol. This scheme, as envisaged by Elsden, is shown opposite.

Role of Acetyl Phosphate.

Lipmann (1939) found in experiments with Bacterium delbrückii that phosphate was necessary for the oxidation

of pyruvate and that during the oxidation, adenylic acid was phosphorylated. He suggested that the pyruvic acid was phosphorylated and dehydrogenated to acetyl phosphate, which in turn donated its phosphate to adenylic acid and yielded acetic acid. Synthetic acetyl phosphate was found to transfer phosphate to adenosine diphosphate (ADP) with the formation of adenosine triphosphate (ATP). Lipmann (1940), using enzyme preparations of B. delbrückii, isolated a compound with similar properties to synthetic acetyl phosphate. The chemistry, determination and synthesis of acetyl phosphate have been described by Lipmann and Tuttle (1944). Koepsell and Johnson (1942) detected acetyl phosphate as a product in the anaerobic dissimilation of pyruvic acid by cell-free extracts of Cl. butylicum. Kalnitsky and Werkman (1943) found that cell-free preparations of Esch. coli attacked pyruvic acid anaerobically with the production of CO_2, acetic, formic, lactic and succinic acids. Inorganic phosphate, Mn^{++} and DPN were found to be components of the enzyme system. Otter and Werkman (1943) studied the anaerobic fission of pyruvic acid by cell-free preparations of Esch. coli and found that acetyl phosphate accumulated in the absence of ADP, and on the addition of ADP this acetyl phosphate disappeared and there was synthesis of ATP. Two new reactions producing acetyl phosphate were found by Bornstein and

Barker (1940), as pointed out by Stadtman and Barker (1948), during the study of the conversion of ethanol and acetate to caproate by an enzyme preparation of Cl. kluyveri. Acetaldehyde oxidation required inorganic phosphate. It could occur aerobically with methylene blue as a carrier, or anaerobically as a dismutation, and resulted in the formation of acetyl phosphate. The second reaction observed was the phosphoroclastic fission of acetoacetate which also gave acetyl phosphate.

Thus it appeared that acetyl phosphate was a key compound in pyruvate metabolism in several bacteria. Ochoa, Peters and Stocken (1939) studying pyruvate oxidation in brain, concluded that acetyl phosphate was not an intermediate in pyruvate oxidation by this tissue. They suggested that pyruvic acid is oxidized firstly to some other intermediate which may in turn yield acetyl phosphate. Attempts to show an acetyl donor function of acetyl phosphate were unsuccessful. Kaplan and Lipmann (1948b) found that acetate, ATP and a dialysed extract of Esch. coli produced pyruvic acid. Synthetic acetyl phosphate did not take part in this reversal of the phosphoroclastic fission of pyruvate. Strecker, Wood and Krampitz (1950) and Strecker (1951) described isotopic experiments which showed that acetyl phosphate was not utilized when formate was fixed by extracts of Esch. coli, and they con-

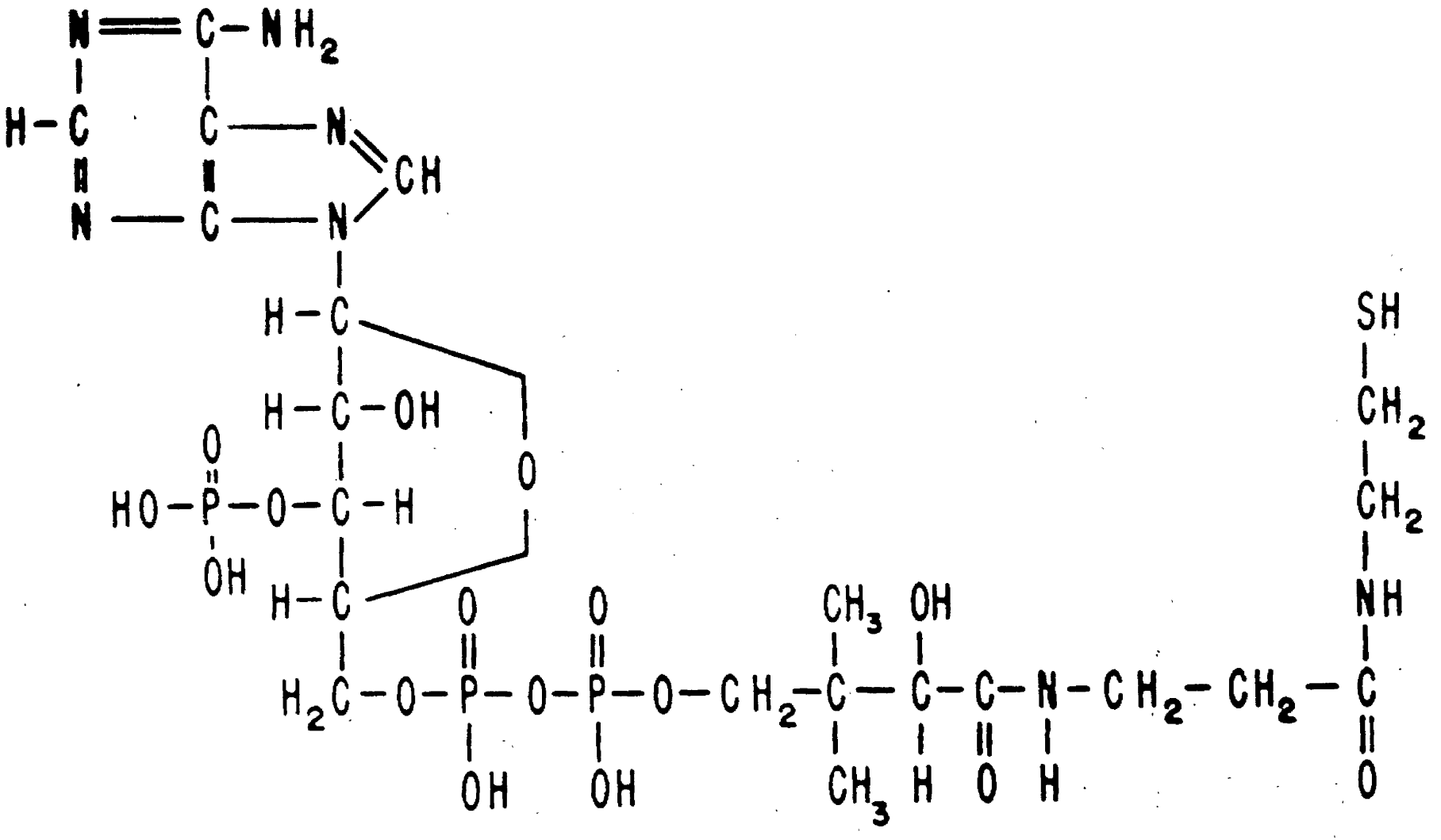
cluded that some other factor accepted the acetyl group from pyruvic acid, and the phosphoroclastic fission was represented thus:

$$\text{Pyruvate} + X \rightleftharpoons \text{Acetyl-}X + \text{Formate}$$

$$\text{Acetyl-}X + HPO_4^{--} \rightleftharpoons \text{Acetyl Phosphate} + X.$$

Discovery of Coenzyme A.

Pigeon liver homogenates were shown to acetylate sulphanilamide when respiring (Lipmann 1945). Acetate, acetoacetate and pyruvate all increased the acetylation, whereas acetyl phosphate was found to be incapable of furnishing acetyl groups. This enzymatic condensation of acetate and sulphanilamide was due to an enzyme system containing a dissociable coenzyme (Lipmann and Kaplan, 1946). The coenzyme was given the name Coenzyme A, and was found to be widely distributed. It was detected in high concentrations in liver, brain, pigeon breast muscle, kidney, spleen, pancreas, and red blood cells, and was not identifiable with any of the other known coenzymes. Lipmann (1947) reported that this coenzyme was a pantothenic acid derivative, while Lipmann, Kaplan and Novelli (1947) showed that it contained pantothenate (11%), phosphorous (9%), adenine (18%), pentose (22%) and some cystine. They established its presence in Lactobacilli, Propionibacterium, Esch. coli and Clostridia; in wheat



COENZYME A

germ, green peas, and in other plant materials. Fresh liver showed a high concentration of CoA, and little or no free pantothenic acid. During autolysis the coenzyme A concentration decreased with a corresponding increase in the pantothenic acid content of the tissue. It appeared that most, if not all, the pantothenate of the tissues was linked in the coenzyme, and this was later confirmed by Novelli, Kaplan and Lipmann (1949). Novelli and Lipmann (1947) added pantothenic acid to deficient cells of Proteus morganii and noted a large and rapid increase in their coenzyme A content. A method of assay of CoA was devised by Kaplan and Lipmann (1948a), who used it in a detailed study of the coenzyme A content of animal tissues, microorganisms and plants. It was found that the coenzyme was a general constituent of living organisms. Lipmann, Kaplan, Novelli and Tuttle (1950) isolated CoA from fresh hog liver and analysed it. From their results and those obtained from its enzymatic degradation by Novelli, Kaplan and Lipmann (1950) a possible structure was assigned to the coenzyme. The structure of CoA which is now accepted is given opposite and is due to Baddiley, Thain, Novelli and Lipmann (1953). A method of purifying the coenzyme using ion exchange chromatography has been devised by Stadtman and Kornberg (1953).

Role of Coenzyme A.

Dorfman, Berkman and Koser (1942) grew cells of P. morganii on a medium deficient in pantothenic acid and studied the effect of added pantothenate on the metabolism of these cells. They found that the main effect was on pyruvate oxidation, and concluded that pantothenate was involved in pyruvic acid metabolism. These results were confirmed by Mills (1943). Olson and Kaplan (1948) found that the CoA content of rat and duck liver, and the ability to utilize pyruvate, decreased when animals were fed a pantothenic deficient diet. Injection of pantothenate increased the CoA content, and also the utilization of pyruvate.

Kaplan and Lipmann (1947) obtained a protein fraction from pigeon liver which contained an acetylating system. Using acetic acid as the only acetyl precursor, and requiring CoA as part of the system, this fraction could acetylate sulphonamide and hydroxamic acid. Using an identical preparation from pigeon liver, Goodak and Lipmann (1948) found that acetoacetate was synthesized from acetate and ATP. CoA was also a component of this reaction, the rate of which depended on the CoA content. The addition of sulphanilamide inhibited the acetylation of acetic acid, and indicated that there was a common precursor in these acetylation reactions. Stern and

Ochoa (1949), reviewing the findings of Kaplan and Lipmann (1948a) and Soodak and Lipmann (1948), emphasized this point. The breakdown of carbohydrate and fatty acids leads to a common acetyl derivative, which through condensation with oxaloacetate forms citric acid, and is then oxidized through the tricarboxylic acid cycle. Pigeon liver extracts readily form citrate from acetate and oxaloacetate in the presence of CoA, ATP and Mg^{++}. Oxaloacetate depressed the synthesis of acetoacetate and the acetylation of sulphanilamide, indicating that the same acetyl derivative was probably involved in the three acetylation reactions.

Novelli and Lipmann (1950) prepared a cell-free extract from Esch. coli which could synthesize citrate from acetic and oxaloacetic acid. They found that CoA was required to 'activate' the acetate, and that acetyl phosphate was also used. Stern and Ochoa (1950) obtained two enzymes from pigeon liver and Esch. coli which in the presence of acetic acid, ATP, oxaloacetate, CoA, and Mg^{++} formed citric acid. They postulated the first step to be the formation of an 'activated acetate' closely related to acetyl phosphate, which then transferred the acetyl group to oxaloacetate.

$$\text{Acetic Acid} + \text{ATP} \longrightarrow \text{'Active Acetate'} \xrightarrow[\text{acetic}]{\text{Oxalo-}} \text{Citrate}$$

Korkes, Stern, Gunsalus and Ochoa (1950) found that enzyme preparations from Esch. coli and Strep. faecalis gave acetyl phosphate, lactate and CO_2 from pyruvate in the presence of phosphate, DPN^+, Mg^{++}, DPT, lactic dehydrogenase, and yeast extract. CoA was required, and if phosphate was omitted from the reaction, and oxaloacetate and the condensing enzyme added, then citrate was synthesized. They postulated that the following reactions were taking place, and suggested that the coenzyme might be CoA

Pyruvate + Coenzyme ⟶ Acetyl-Coenzyme + CO_2

+ Phosphate → Acetyl Phosphate + Coenzyme

+ Oxaloacetate → Citrate + Co-enzyme.

During the study of the synthesis of fatty acids in extracts of Cl. kluyveri, it was observed that addition of inorganic arsenate completely inhibited the reduction of acetyl phosphate to butyrate. This inhibition was shown to be due to complete and almost instantaneous decomposition of acetyl phosphate in the presence of arsenate (Stadtman and Barker, 1950). The similarity of this catalytic effect of arsenate on the hydrolysis of acetyl phosphate to the effect of arsenate on the hydrolysis of glucose-1-phosphate by the glucose-transferring enzyme obtained from Pseudomonas saccharophila (Doudoroff, Barker

and Hassid, 1947) led to the postulation that the arsenolysis of acetyl phosphate was due to an acetyl transferring enzyme catalyzing reaction (1)

Acetyl Phosphate + X $\rightleftharpoons$ Acetyl-X + Phosphate ..... (1)

Acetyl-X + Arsenate $\rightleftharpoons$ Acetyl-Arsenate + X ..... (2)

Acetyl-Arsenate + H_2O $\xrightarrow{\text{Spontaneous}}$ Acetate + Arsenate .. (3)

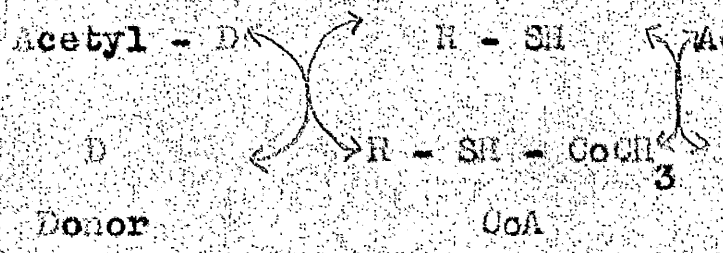
Substitution of arsenate for phosphate gives reaction (2), which results in the formation of acetyl-arsenate, a compound undergoing spontaneous hydrolysis (3). Stadtman, Novelli and Lipmann (1951) showed that this arsenolysis was dependent on CoA, and they suggested that coenzyme A was in actual fact the acetyl acceptor X, referred to in reaction (1). Thus the enzyme was visualised as catalyzing the transfer of the acetyl group from acetyl-CoA to orthophosphate, and was called phosphotransacetylase (or more briefly, transacetylase).

Acetyl - Phosphate + CoA $\rightleftharpoons$ Acetyl - CoA + Phosphate

Chantrenne and Lipmann (1950) following Stadtman's (1950) discovery of transacetylase amended the scheme proposed by Korkes et al. (1950) to read Coenzyme A for coenzyme.

Final proof that the 'active acetate' complex was in actual fact acetyl - CoA came when Lynen and Reichart (1951) isolated 'activated acetate' from bakers' yeast grown on very dilute ethanol or acetic acid. In the

presence of pigeon liver enzyme, it acetylated sulphanilamide. They showed that it was CoA acetylated in the sulphur group, and described its function as that of a transacetylase. It transfers the acetyl group from a donor (e.g., acetyl phosphate, acetic acid, citric acid) to an acceptor (e.g., oxaloacetate, sulphanilamide, choline).



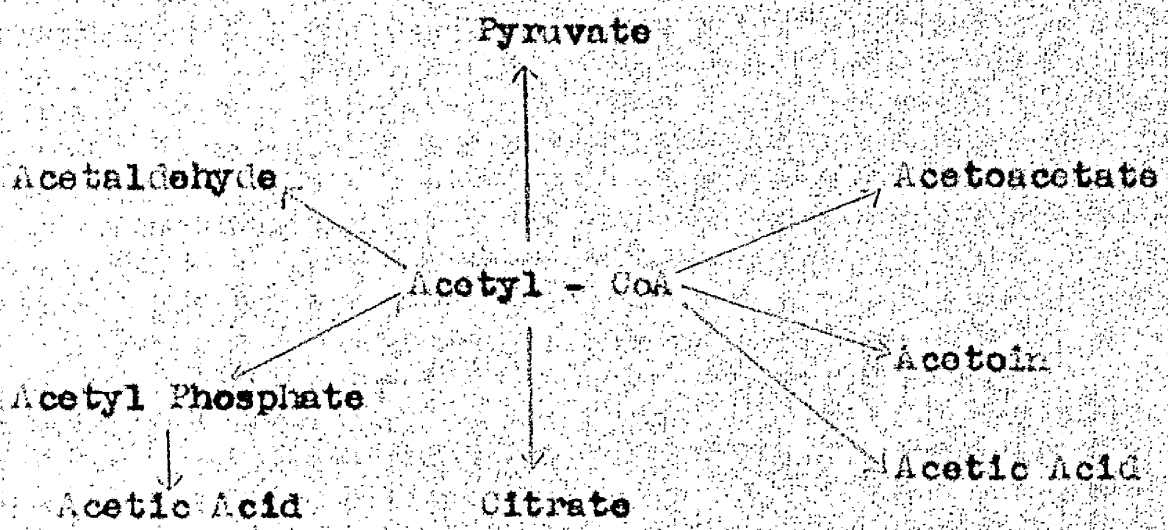
Ochoa, Novelli, Stadtman and Lipmann (1950) separated by acetone fractionation the acetyl donor, (acetate + ATP) and the (sulphanilamide, acetoacetate and citrate synthesis) acceptor systems. Using Stadtman's (1950) phosphotransacetylase from Cl. kluyveri they coupled the activation of acetyl phosphate with the acceptor systems, and found that acetyl phosphate liberates acetyl which condenses with oxaloacetate to give citrate, and also that two molecules of labelled acetyl phosphate gave rise to acetoacetic acid. Stern and Ochoa (1951) gave the following scheme for citric acid synthesis:

Acetate + ATP $\rightleftharpoons$ Acetyl Phosphate + ADP ..... (1)

Acetyl Phosphate + CoA $\rightleftharpoons$ Acetyl - CoA + Phosphate . (2)

Acetyl - CoA + Oxaloacetate $\rightleftharpoons$ Citrate + CoA ..... (3)

Functions of Acetyl-CoA



Reaction (2) is catalyzed by phosphotransacetylase, and reaction (3) by the condensing enzyme. The enzyme for reaction (1) has been isolated and partially purified from Esch. coli and Streptococcus haemolyticus by Rose, Grunberg-Manago, Korey and Ochoa (1954).

Korkes (1951) and Korkes, Del Campillo, Gunsalus and Ochoa (1951) studied pyruvate as the acetyl donor. They found that the dismutation of pyruvate to lactate, acetyl phosphate, and CO_2 required four enzymes with DPN, DPT, CoA, Mg^{++} or Mn^{++} as co-factors. The reactions take place thus:

$$\text{Pyruvate} + DPN^+ + CoA \xrightarrow{A.\ B.} \text{Acetyl} - CoA + CO_2 + DPNH + H^+$$

$$\text{Acetyl} - CoA \xrightarrow{C.} \text{Acetyl Phosphate} + CoA$$

$$\text{Pyruvate} + DPNH + H^+ \underset{\leftarrow}{\xrightarrow{D.}} \text{lactate} + DPN^+.$$

Enzyme C is phosphotransacetylase, and enzyme D is lactic dehydrogenase. The two enzymes A and B have been isolated from Esch. coli, and acetyl CoA was isolated as the end-product of their action on pyruvate.

Acetaldehyde Dismutation.

Stadtman and Barker (1949) obtained extracts of Cl. kluyveri which catalyzed the oxidation of acetaldehyde in the presence of orthophosphate to acetyl phosphate.

$$CH_3CHO + HPO_4^{--} + \tfrac{1}{2}O_2 \longrightarrow CH_3COOPO_3^{--} + H_2O$$

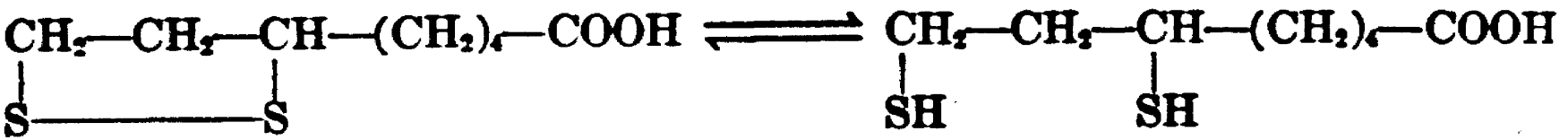
This is analogous to the oxidation of pyruvate by extracts of B. delbrückii reported by Lipmann (1939). The stages of pyruvate oxidation have now been elucidated, and the reactions are shown in the previous diagram. A similar scheme was suggested for acetaldehyde dismutation by Burton and Stadtman (1953).

$$\text{Acetaldehyde} + DPN^+ + CoA \rightleftharpoons \text{Acetyl - CoA} + DPNH + H^+ \quad ..(1)$$

$$\text{Acetyl - CoA} + HPO_4^{--} \rightleftharpoons \text{Acetyl-Phosphate} + CoA \quad .....(2)$$

$$\text{Acetaldehyde} + H^+ + DPNH \rightleftharpoons \text{Ethanol} + DPN^+ \quad .....(3)$$

The enzymes involved would be aldehyde dehydrogenase for (1), transacetylase for (2) and alcohol dehydrogenase for (3). Experimental support for this scheme has been provided by Burton (1951, 1952), who succeeded in purifying the aldehyde dehydrogenase of Cl. kluyveri. He showed that all three enzymes are required to convert acetaldehyde to acetyl phosphate; also DPN, CoA, inorganic phosphate, and glutathione are required as cofactors. Spectrophotometric experiments, Burton and Stadtman (1953), with purified enzymes supported the sequence of reactions given. All these studies on acetaldehyde oxidation have been carried out on Cl. kluyveri. Pinchot and Racker (1951) studying the oxidation of ethanol and acetaldehyde by extracts of Esch. coli showed that acetaldehyde



α-lipoic acid

reduced α-lipoic acid

oxidation, but not the ethanol system, was CoA dependent.

Lipoic Acid.

O'Kane and Gunsalus (1948) studied the oxidative activities of Strep. faecalis cells, harvested from several growth media and found that they exhibited a nutrient requirement for the oxidation of pyruvate. This could not be satisfied by any of the known vitamins or co-enzymes, and was called the "pyruvate oxidative factor" (POF). A study of its properties showed a similarity between POF and several unidentified growth factors found by Guirard, Snell and Williams (1946), Stockstad, Hoffman, Hogan, Firdham and Jukes (1949), Snell and Broquist (1949) for Tetrahymena geleii, and by Kline and Barker (1950) for Butyribacterium rettgeri. Reed, De Busk, Gunsalus and Hornberger (1951) isolated a crystalline substance, lipoic acid, which possessed POF activity. Lipoic acid, a cyclic disulphide, and its conjugates form a group of substances active in pyruvate oxidation. The structure of lipoic acid is given opposite.

Barner and O'Kane (1952) with Strep. faecalis showed that although lipoic acid is needed for the oxidation of pyruvate, it is not required for the oxidation of ethanol or acetaldehyde to acetic acid. Thus it was accepted that the site of action of lipoic acid was between

pyruvic acid and acetyl CoA. From Esch. coli extracts, Korkes *et al.* (1951) obtained two fractions, A and B, which were required to convert pyruvate to acetyl CoA. Thus at least two enzymes are required in this reaction; also DPT, DPN, and CoA are required as cofactors. The exact nature of the decarboxylase reaction, and the DPN reductant of this system, were unknown.

$$\text{Pyruvate} + DPN^+ + CoA \longrightarrow \text{Acetyl - CoA} + CO_2 + DPNH + H^+$$

As a working hypothesis for the initial steps of decarboxylation and dehydrogenation, Gunsalus (1953) suggested the following series of reactions:

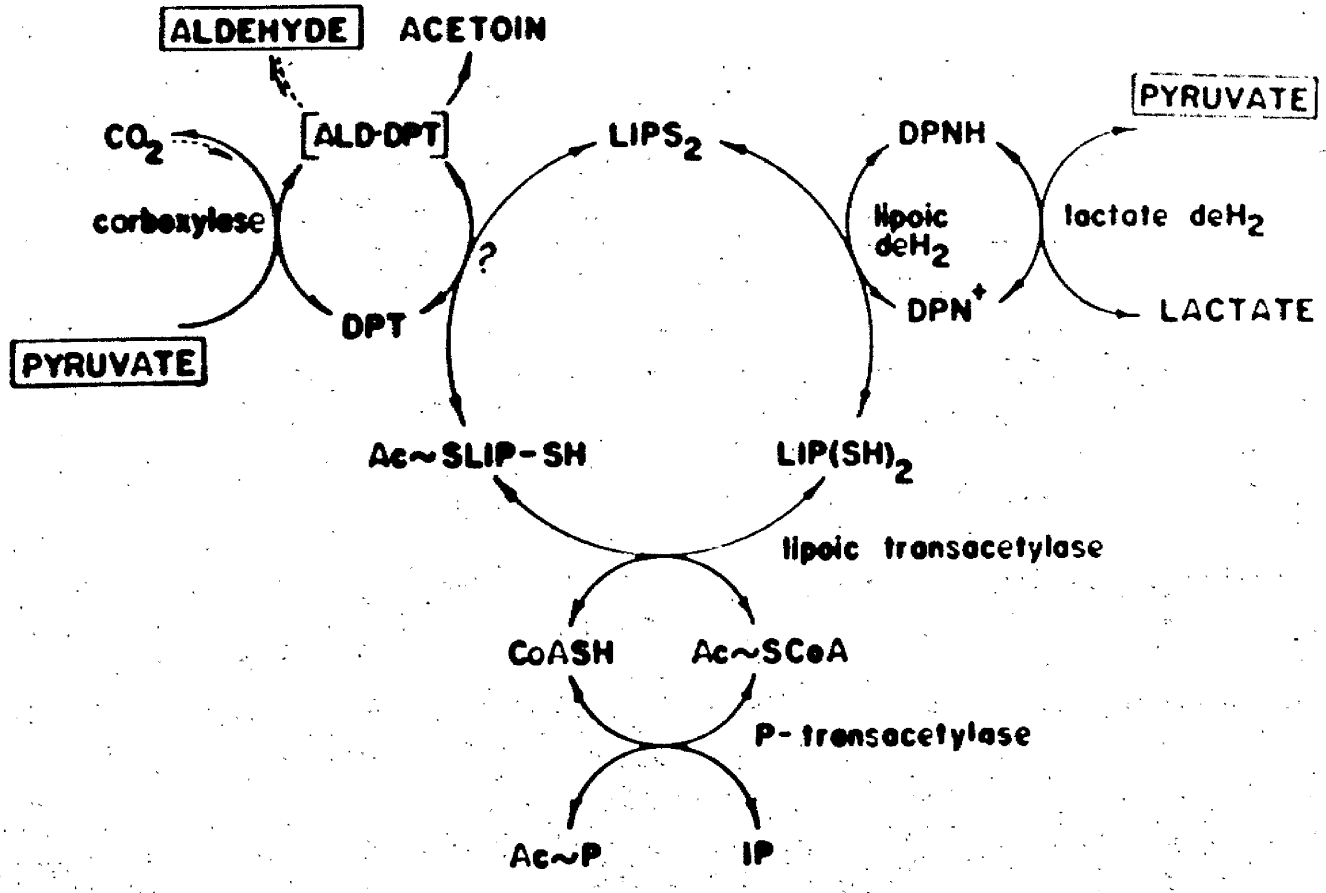
$$\text{Pyruvate} \longrightarrow \text{Aldehyde - DPT} + CO_2$$

$$\text{Aldehyde - DPT} + \text{Carrier} \rightleftharpoons \text{DPT} + \text{Aldehyde - Carrier}$$

$$\text{Aldehyde - Carrier} + DPN^+ \rightleftharpoons \text{Acyl - Carrier} + DPNH + H^+$$

Kolin and Gunsalus (1951) showed that extracts of Strep. faecalis form acetoin from pyruvate via the acetolactate pathway of Juni (1952). This system requires DPT and Mg^{++}, but not lipoic acid (O'Kane, 1950, Kolin and Gunsalus, 1951). Thus the function of lipoic acid must follow the decarboxylation of pyruvate.

Peters, Sinclair and Thompson (1946) found that brain pyruvate oxidase could be specifically inhibited by Lewisite, and this inhibition could be reversed by 2:3-dimercaptopropanol (British Anti-Lewisite or BAL). They


ALDEHYDE
ACETOIN
ALD-DPT
CO2
carboxylase
DPT
PYRUVATE
?
LIPS2
DPNH
lipoic deH2
DPN+
PYRUVATE
lactate deH2
LACTATE
Ac~SLIP-SH
LIP(SH)2
lipoic transacetylase
CoASH
Ac~SCoA
P-transacetylase
Ac~P
IP

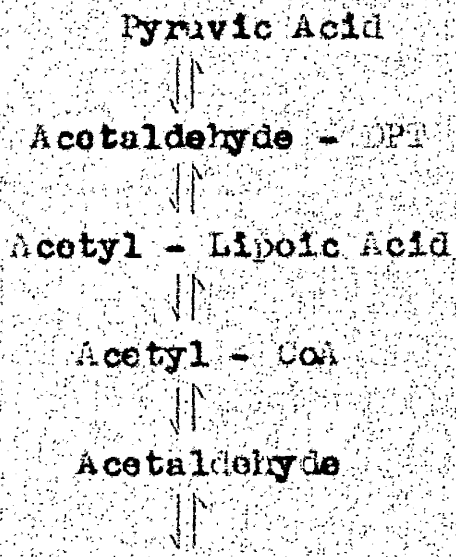
suggested that a cyclic dithiol must be a cofactor of the pyruvate oxidation system. Gunsalus (1953) presented results which showed that arsenate inhibited lipoic acid function, and that this could be reversed by dithiols, but not by monothiols. Thus lipoic acid must function in its dithiol form.

Chin and Gunsalus (1954) reported that fraction A of the pyruvate dehydrogenase system of Esch. coli could catalyze the formation of acetoin from acetyl phosphate in the presence of reduced lipoic acid ($Lip(SH)_2$), phosphotransacetylase, CoA, DPT, Mg^{++} and acetaldehyde. Gunsalus (1954) combined the fact that lipoic acid functions as a dithiol, between the decarboxylation of pyruvate and the CoA site, with these findings and drew up the scheme given opposite. Many of these steps are well established. It seems strange that reaction (4) involving DPT, the first cofactor known to function in keto acid metabolism, is the one whose reaction is least understood. Reaction (3) involves reductive acetylation of lipoic acid. Chin and Gunsalus (unpublished results) added TPT and Mg^{++} with substrate amounts of DL - α lipoic acid to pyruvic acid in the presence of Esch. coli fraction A and found that 6-acetyl-monomercapto lipoic acid accumulated. On the addition of transacetylase, and inorganic phosphate, acetyl

phosphate accumulates and only 6,8-dimercapto octanoic acid is found, i.e., reduced lipoic acid. The enzymatic acetylation of reduced lipoic acid, reaction (2), has been demonstrated by linking the transfer of acetyl from phosphate to CoA with a system containing a lipoic transacetylase, which transferred the acetyl to reduced lipoic acid. Hager and Gunsalus (1953) showed the presence of lipoic acid dehydrogenase, reaction (1), in Esch. coli fraction B, using substrate amounts of reduced lipoic acid in the presence of pyruvate and lactic dehydrogenase.

This work has mostly been carried out using Esch. coli extracts, and must not be applied generally to bacterial metabolism. Hager, Diller and Lipmann (1954) have shown that L. delbrückii oxidizes pyruvate to acetyl phosphate and CO_2 by a system where apparently neither CoA nor lipoic acid is implicated. Removal of CoA by Dowex-1 (Chantrenne and Lipmann, 1950) did not affect the rate of oxidation, and there was no evidence of arsenic sensitivity. A flavin-adenine dinucleotide was isolated from the system, and the rate of oxidation was proportional to its concentration. O'Kane (1954) showed that the Proteus pyruvate system required only cocarboxylase and Mg^{++}. There was no evidence for any other cofactor such as lipoic acid, CoA, DPN, or TPN. Barker

Proposed Series of Reactions Leading from Pyruvate to Ethanol in Escherichia Coli.



(1954) worked with Butyribacterium rettgeri and found that it too did not require lipoic acid for pyruvate oxidation. It did, however, require lipoic acid for growth when lactate was used as the fermentable substrate (Kline and Barker, 1950; Kline, Pine, Gunsalus and Barker, 1952). When lactate was replaced by glucose or pyruvate, lipoic acid was not needed, and it was not synthesized by the cells during growth. When the cells were grown on a mixture of glucose and lactate, without lipoic acid, they grew readily on the glucose but were completely unable to decompose the lactate. Thus lactate but not pyruvate utilization was inhibited by arsenate. Hence lipoic acid is required either in the conversion of lactate to pyruvate, or in the transport of lactate into the cell.

Pyruvate to Ethanol in Esch. coli.

From our present knowledge we can propose that the production of ethanol from pyruvate follows the pathway shown opposite. All the reactions have been shown in Esch. coli. The oxidation of acetaldehyde to acetyl-CoA has not been reversed.

EXPERIMENTAL

Experimental Methods.

The organism used in this work was Escherichia coli, N.C.T.C. 5923. The stock culture was maintained by monthly subculture onto nutrient agar slopes. Liquid cultures for use as inocula were grown in a glucose-ammonium salt medium containing KH_2PO_4 5.4 g., $(NH_4)_2SO_4$ 1.2 g., $MgSO_4.7H_2O$ 0.4 g., and glucose 12 g. per litre, adjusted to pH 7.1 by the addition of 5N NaOH. The phosphate buffer used contained KH_2PO_4 9 g./l., and except where otherwise stated was adjusted to pH 7.1. Growth, washed cell suspension and cell-free extract experiments, with the exception of the spectrophotometric experiments, were carried out at 37°C. Solutions were sterilized by boiling, by autoclaving in an autoclave or pressure cooker, or by passage through a sterile Seitz filter. All glassware used was cleaned by boiling in nitric acid (10% v/v) and rinsed in glass distilled water. Except when media was being prepared in bulk (20-40 litres), glass distilled water was used in all media and solutions.

Growing cultures were aerated by the passage of a gentle stream of air through the media. Semi-anaerobic conditions were obtained by growing cultures in flasks, which were filled with media almost up to the non-absorbent

ERRATUM: p.47, line 6.

For 'calcium sulphate' read 'calcium chloride'.

cotton wool plug. Anaerobic cultures were grown in a Wildes-Macintosh jar. Cell suspensions and cell-free extracts were tested under anaerobic conditions, obtained by using an atmosphere of N_2 in the tubes.

The calcium phosphate gel was prepared by the method of Kellin and Hartree (1938). Calcium sulphate and sodium phosphate are mixed. The pH of the suspension is adjusted to 7.6 by the addition of acetic acid. The precipitate is washed with water several times, by decanting. It is then centrifuged, washed twice with distilled water, and suspended in a little distilled water. The gel was always prepared the day before use.

Analytical Methods.

Glucose was estimated by the method of Nelson (1944). The microdiffusion method of MacLeod (1949) was used for ethanol determinations. Lactic acid was estimated by the Mullen and Noble (1953) modification of the Barker and Summerson (1941) method. Pyruvic acid estimations were carried out by the method of Friedemann and Haugen (1943) and also by a modification of this method (Dawes and Holms, 1954). Friedemann's (1938) distillation method was used for acetic acid determinations. Total volatile acids were estimated by the method described by Werkman (1942). The principle of all these methods

has been briefly described in the previous 'Analytical Methods' section.

Acetyl phosphate was estimated by the method of Lipmann and Tuttle (1945). This method utilizes the reaction of acyl phosphates with hydroxylamine. The acyl group is converted into the hydroxamic acid

$$R.COO.PO_3^{=} + NH_2OH \longrightarrow R.CO.NHOH + HPO_4^{=}.$$

This hydroxamic acid then forms with trivalent iron (ferric chloride) a bright purplish colour which is a measure of the original acyl phosphate. This colour was found to be linearly proportional to the concentration of acyl phosphate only within the range of 1 to 2.5 µmoles/ml.

The protein in cell free extracts was determined by the colorimetric Stickland reaction (1951). Sodium hydroxide is added to the extract, followed by copper sulphate, which forms a precipitate. This is broken up with a glass rod and removed by centrifugation. The supernatant is purple, the intensity of the colour being linearly proportional to the protein present in the extract over the calibrated range of 0-0.65 mg. protein nitrogen.

The purity of ATP preparations was determined by Elmor and Stocken's (1950) modification of the method of Berenblum and Chain (1938). The ATP is subjected to complete acid hydrolysis by heating with H_2SO_4, to give

the total phosphorus content. The acid labile phosphorus is split off by a ten-minute hydrolysis with HCl. The ratio of these two phosphorus concentrations, corrected for the inorganic phosphate in the original solution, is a criterion of the purity of the ATP. Theoretically the acid labile phosphorus should represent 66.6% of the total phosphorus. In practice values of about 60% were obtained with fresh preparations. After storage for a year, this value fell to 40%.

The purity of DPN preparations was estimated by the spectrophotometric method of Lepage (1947). In this method, the DPN is reduced by sodium hydrosulphite and the increase in absorption due to the reduced DPN is measured at 340 mμ with a 1 cm. cell in a Unicam SP 500 spectrophotometer.

Pure dihydrocozymase was prepared from DPN by the method of Ohlmeyer (1938). The DPN is reduced by sodium hydrosulphite in a Warburg manometer under an atmosphere of 5% CO_2 - N_2 95%. The reduced DPN is precipitated by ethanol, redissolved in methanol, and reprecipitated by the addition of an ethanol-ether mixture. The reduced DPN was studied in a Unicam SP 500 spectrophotometer. From the absorption spectrum it was found to be 94% pure, giving an E 260/E 340 ratio of 2.7 instead of the theoretical value of 2.5 to 2.6. A commercial preparation of

dihydrocozymase, obtained from Boehringer and Soehne (Germany), was found to be 87% pure, and gave an E 260/E 340 ratio of 3.2.

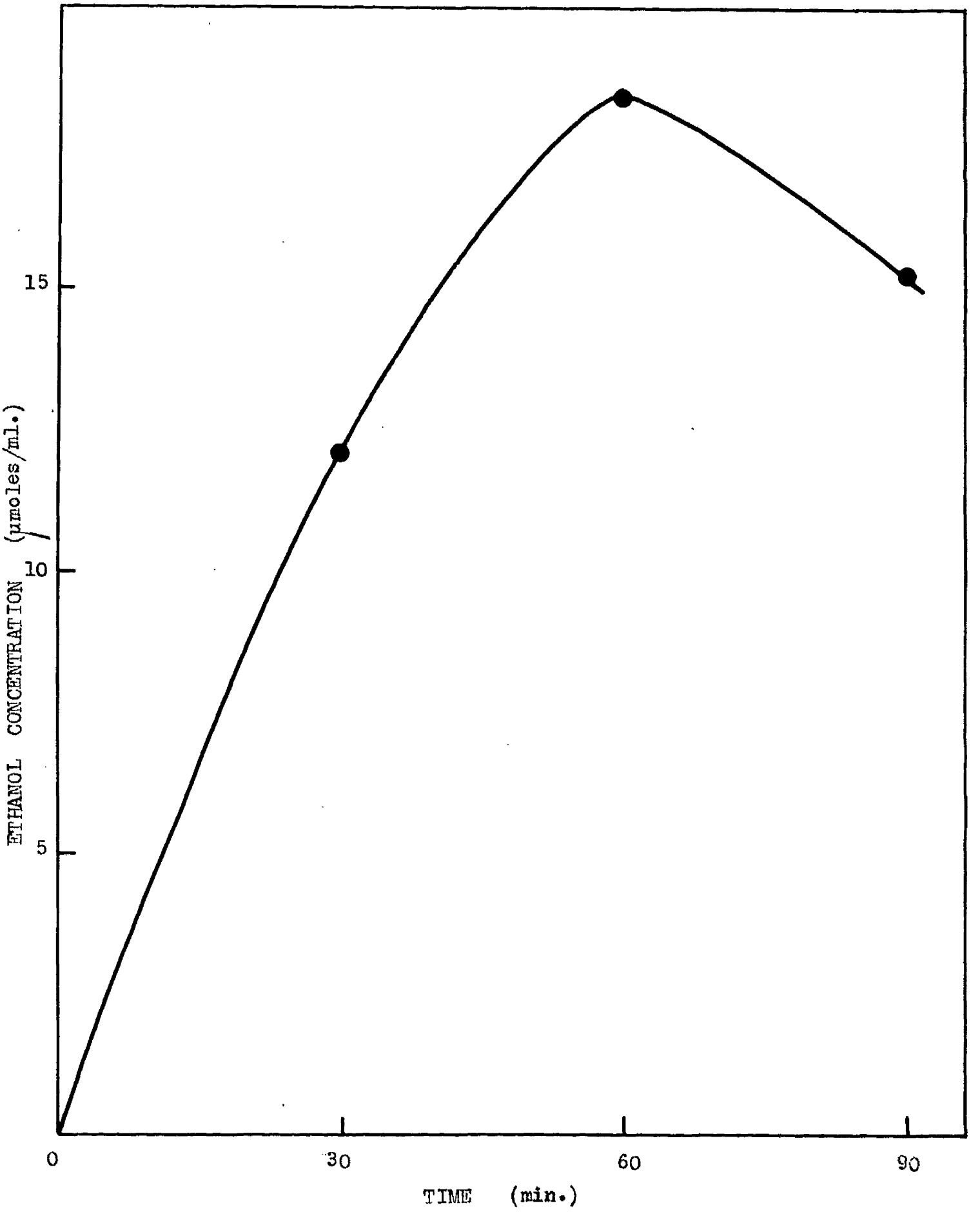
Preparation of cell free extracts.

The details of these methods will be described in the text. Cells were disrupted by grinding with alumina as described by McIlwain (1948). In the preparation of acetone dried powders, 30 ml. cell suspension were added to 90 ml. chilled acetone. After standing for about 30 min., the powder was filtered through a Buchner funnel, washed with a 50 : 50 acetone ether mixture, and finally with ether. Other cell free extracts were prepared by shaking cells with glass beads in a Mickle shaker, by subjecting the cells to the vibrations produced by an ultrasonic generator, B. 7562, 250-2000 kc/s, at 1000 kc/s, and by crushing in a Hughes' bacterial press (Hughes, 1951). Cell suspensions were freeze-dried. The freeze-dried powder was ground with alumina before extraction (Korkes, 1951). Stadtman (1949) dried Clostridium kluyveri in a petri dish in vacuo over $CaCl_2$. Two attempts to dry Esch. coli by this means were unsuccessful. The result was a brown resinous mess. Hochster and Quastel (1951) in the preparation of yeast extracts of high fermentative power, added nicotinamide to their extracting

fluid, a procedure which inhibits the enzyme diphosphopyridine nucleotidase, and prevents the disappearance of DPN from the extracts. This was adopted in the preparation of some cell-free extracts in the present work.

[illegible].

Fig. 4. Production of ethanol from glucose (25 μmoles/ml.) by a non-proliferating cell suspension of Escherichia coli under an atmosphere of H_2.


15
10
5
ETHANOL CONCENTRATION (µmoles/ml.)
0
30
60
90
TIME (min.)

Production of Ethanol from Glucose.

The following method was adopted as the standard procedure in testing cell suspensions, or cell-free extracts, for the ability to produce ethanol from glucose. 6 x 1 in. Pyrex tubes were used. 5 ml. cell suspension, usually with a dry weight of 21-25 mg./ml., were set up with 5 ml. phosphate buffer, 0.06 ml. $MgSO_4 7H_2O$ (10%) and glucose solution to give a final concentration of 25 μmoles/ml., the total volume being made up to 15 ml. A control was set up with the glucose replaced by water. The tubes were stoppered with rubber bungs which carried inlet and outlet glass tubing, so that the tubes could be flushed with nitrogen from a cylinder. The tubes were placed in a bath at 37°, and samples withdrawn at time intervals of, usually, 0, 30, 60, 90 and 120 min. A typical curve of ethanol production from glucose by Esch. coli is shown in Fig.4. This shows that the ethanol concentration reaches a maximum after about 60 min. and then begins to fall. The activity of a batch of cells was usually expressed as μmoles ethanol/mg. dry weight of cells/ml. produced from 25 μmoles glucose in 60 min. The normal batch of active cells had an activity of approx. 2.4 units.

Table 6.

The effect of different growth media and conditions on the ability of *Escherichia coli* to product ethanol from glucose.

Activity = μmoles/mg. dry weight cells/ml ethanol produced from 25 μmoles/ml. glucose in 60 min.

Growth Medium	Conditions	Activity
Complex Peptone, glucose, yeast extract and vitamins	Aerated during growth	0.25
	Unaerated during growth	1.62
	Semi-anaerobic during growth	2.35
	Anaerobic during growth	2.4
Simple-defined Glucose-ammonium salt	Aerated during growth	0.12
	Unaerated during growth	0.63
	Semi-anaerobic during growth	1.22
'Simplified' Glucose-ammonium salt with supplements of peptone, yeast extract and vitamins	Fresh cells	2.44
	Cells stored at 0° for 14 days	2.38
	Cells grown with pantothenate added to medium	2.61
	Cells grown without vitamin salts solution	2.39

The effect of different growth conditions on the ability of Escherichia coli to produce ethanol from glucose.

Cultures were grown initially on a complex medium containing peptone 10 g., yeast extract 10 g., KH_2PO_4 5 g., glucose 10 g., and 100 ml. vitamin salt solution, per litre, at a pH value of 7.1. The vitamin salt solution contained thiamin hydrochloride 100 mg., $MnSO_4.4H_2O$ 2 g., $MgSO_4.7H_2O$ 4 g., NaCl 100 mg., $FeSO_4.7H_2O$ 100 mg. and ascorbic acid 500 mg. in 100 ml. distilled water. One litre of this medium was inoculated with 10 ml. of a 16 hour culture, grown in the same medium, and incubated at 37° for 16 hours. The cells were harvested, washed twice with phosphate buffer, and suspended in the same buffer. The activities of cells grown with aeration, without aeration, semi-anaerobically and anaerobically are shown in Table 6.

Cultures were then grown on the simple defined glucose-ammonium-salt medium used for the stock liquid cultures. Cells grown with aeration, without aeration and semi-anaerobically for 16 hours at 37° were tested, and their activities are shown in Table 6.

A range of media containing decreasing amounts of peptone and yeast extract was made up. The peptone-yeast extract solution contained peptone 10 g., yeast extract 10 g., and KH_2PO_4 5 g. per litre, and was adjusted to

Table 7.

Production of ethanol from glucose by cells grown in media containing decreasing amounts of peptone and yeast extract.

Solution	(1)	(2)	(3)	(4)	(5)	(6)
Peptone-yeast	90	70	50	30	10	1
Phosphate-ammonia	1.0	21	41	61	81	90
Glucose	10	10	10	10	10	10
Vitamin salt	1.0	1.0	1.0	1.0	1.0	1.0
Inoculum	1.0	1.0	1.0	1.0	1.0	1.0

Activity — Ethanol produced from glucose (25 µmoles/ml.) by washed cell suspensions from the above media

	(1)	(2)	(3)	(4)	(5)	(6)
Ethanol (µmoles/ml.)	19.6	17.0	20.4	14.4	17.0	9.2
Ethanol (µmoles/10^6 /cells/ml.)	5.0	3.8	4.8	4.9	4.1	5.9

pH 7.1. A 10% (W/V) glucose solution was prepared, also a solution containing KH_2PO_4 9 g. and $(NH_4)_2SO_4$ 2 g. per litre, with pH adjusted to 7.1. These solutions, together with a vitamin salt solution, were sterilized and aseptically added to a series of tubes as shown in Table 7. The inoculum was taken from a 16 hour culture in the simple glucose-ammonium salt medium. The tubes were incubated under semi-anaerobic conditions for 24 hours at 37°. The cultures were then centrifuged, the cells washed twice and then suspended in phosphate buffer. They were tested for ethanol production, and the results are given in Table 7.

As a result of this experiment, cells were grown in media made up of KH_2PO_4 9.0 g., $(NH_4)_2SO_4$ 1.2 g., glucose 12 g., peptone 0.5 g., yeast extract 0.5 g. and vitamin salt solution (100 ml.) per litre. The yield from this medium was 1.3 grams wet weight bacteria per litre. The activity of cells grown in this medium (designated 'simplified medium') is given in Table 6. The cells were stored in suspension at 0° and were retested after 14 days. The vitamin salt solution did not contain pantothenic acid. A fresh batch of cells was grown on the simplified medium with the addition of pantothenic acid (20 mg. per litre) to the vitamin salt solution, and the activity compared with the previous batch. One

difficulty was experienced with this medium. The concentration of metallic ions in the vitamin salt solution caused a dense precipitate with the phosphate of the medium. This did not affect the growth of the cells, but proved an inconvenience when harvesting as the cultures had to be filtered through glass wool before being put through the Sharples centrifuge. The vitamin salt solution was therefore omitted from the medium and the resulting cell suspension tested for activity. The results of this and the two previous tests are given in Table 6.

Procedure for growing cells.

As a result of these preliminary experiments, the following procedure was adopted for growing cultures of Esch. coli in bulk. A solution of KH_2PO_4 36 g. and $(NH_4)_2SO_4$ 4.8 g. in 2,400 ml. water contained in a 5 l. flask was adjusted to pH 7.1 and sterilized by boiling. A sterile solution containing glucose 48 g. and $MgSO_4.7H_2O$ 1.6 g. in 1,600 ml. water was added aseptically followed by 50 ml. of a sterile solution of peptone 10 g. and yeast extract 5 g. per litre. All these solutions were at 37°. The final medium was inoculated with 40 ml. of a fresh liquid culture of Esch. coli and incubated at 37° for 20 hours. The cells were then harvested using a Sharples super centrifuge, washed twice with distilled water and stored as a 100 ml. suspension in the refrigerator.

The usual yield from such media was approx. 1.0 to 1.2 grams wet weight cells per litre. The cells were normally used for about two weeks after harvesting.

'Inactive' cells.

It was possible to grow two batches of cells, under seemingly identical conditions, and for one batch of cells to produce 12 to 15 µmoles ethanol, whereas the other batch only produced about 4 to 6 µmoles ethanol from 25 µmoles glucose. The comparison of the activity of cells tested straight from the Sharples without washing with the activity of those which had been washed twice before testing showed that washing did not affect the activity. Various attempts were made to activate the cells. They were suspended in buffer containing cysteine (25 mg./12 ml.), and in water containing cysteine (25 mg./12 ml.) and incubated for 2 hrs. before testing, to investigate the possibility that some essential enzyme system had become oxidized during the harvesting and washing. They were suspended in complex growth medium (peptone 10 g., yeast extract 5 g., KH_2PO_4 9 g., glucose 12 g. and $MgSO_4.7H_2O$ 0.4 g. per litre) and incubated at 37° overnight. The cell mass (wet weight) doubled in this time, but the cells remained 'inactive'. They were also suspended in glucose-ammonium salt media for four hours, but the har-

Table 8.

The effect of various treatments of 'inactive' cells on their ability to produce ethanol from glucose (25 umoles/ml.).

	Ethanol formation (umoles/ml.)	
	Control	Test
(a) Fresh cells	0	7.14
(b) Cells suspended in cysteine and buffer for 2 hrs.	0	5.88
(c) Cells susponded in cysteine and water for 2 hrs.	0	6.05
(d) Cells suspended in glucose-ammonium salt media for 4 hrs.	5.08	6.65
(e) Fresh cells	0	4.47
(f) Cells suspended in complex media overnight	0	3.4

vested cells were even less active than before. The results are given in Table 8.

The following points were noted about 'inactive' cells. An 'inactive' batch of cells still utilized glucose although they did not produce ethanol. The glucose utilized in 60 mins. by a cell suspension, which only produced 3.4 μmoles/ml. ethanol, was 15 μmoles/ml., a ratio of ethanol : glucose of 0.22 : 1. The usual ratios obtained were 0.67 : 1, 0.7 : 1, 0.73 : 1. In experiments where a low yield of ethanol was obtained, it was always observed that the fermentation gases were suppressed even although the glucose was still being utilized. Cell suspensions had been obtained which could oxidize ethanol even under anaerobic conditions. 'Inactive' cells could not oxidize ethanol either anaerobically or in the presence of air. The problem of why such cells were obtained remains unsolved. When an 'inactive' batch of cells was grown, it was discarded, and a fresh lot grown.

Cell free extracts.

(a) Grinding with alumina. 2 g. wet weight cells were mixed with 5 g. alumina (Griffin & Tatlock Microid Polishing Alumina, Grade 3/50) and the paste chilled. It was then ground with a chilled agate pestle and mortar, mixed with 6 ml. phosphate buffer containing nicotinamide (35

Table 9.

Production of ethanol and pyruvate from glucose (25 umoles/ml.) by cell-free extracts obtained by different methods.

Treatment	Extraction Fluid	Additional Cofactors	Length of Incubation	Pyruvate umoles/l.	Ethanol umoles/ml.
(a) Alumina grinding (2)	Phosphate buffer	-	90	-	3.8
	+ nicotinamide	Yeast extract	90	-	3.2
		-	60	-	5.0
(b) Acetone dried powder	Phosphate buffer	-	60	-	8.8
		Yeast extract	60	-	8.8
	KCl satd.	-	60	-	8.0
(2)	Phosphate buffer	-	60	-	10.0
(3)	do.	-	60	-	5.5
(4)	do.	-	60	-	4.1
(5)	do.	-	60	0	0
		DPN, ATP, CoA	60	0	0
(f) Crushing	Phosphate buffer	-	0	40	0
			60	270	0
			120	444.4	0

mg./l.) and centrifuged. The extract was tested for ethanol production from glucose, with and without the addition of yeast extract (0.1 ml. of a 10% (W/V) solution). The results are given in Table 9.

(b) Acetone-dried powder. The acetone-dried powder was suspended in both phosphate buffer and saturated KCl, to give 2% suspensions. These suspensions were shaken with a microid shaker at 37°, and samples withdrawn over a period of hours. These samples were centrifuged and the extracts tested for ethanol production from glucose. The most active extract was obtained by shaking in either phosphate buffer or saturated KCl for 60 minutes. The activity of the extracts, however, varied from experiment to experiment as Table 9 shows.

(c) Ultrasonic treatment. Cell suspensions were subjected to ultrasonic vibrations for different periods of time, and the centrifuged extracts were tested for ethanol production from glucose. These extracts were inactive. Estimation of pyruvate in the samples, removed from the glucose-extract mixture, revealed that no pyruvate was produced from the glucose. The addition of cofactors, CoA, DPN, DPT and ATP had no effect on the activity.

(d) Hickle shaker. A cell suspension was mixed with glass beads and shaken for 15, 30, 45, 60 mins. in the Hickle shaker. The centrifuged extracts produced no ethanol from

glucose.

(e) Freeze-dried cells. Cell suspensions (25 ml.) were freeze-dried for three to four hours. A 20% (W/V) suspension of the freeze-dried powder was made with phosphate buffer and this was shaken in a 'Micrid' shaker at 37°. Samples were withdrawn after 0, 10, 20, 30 and 60 min., and centrifuged at 0° for 60 minutes. The extracts produced neither pyruvate nor ethanol from glucose. A similar suspension of the freeze-dried powder in phosphate buffer was tested for ethanol production from glucose. This had no activity.

(f) Crushed cells. A wet mass of cells, 6 g., was crushed in the Hughes' press and extracted with 30 ml. phosphate buffer. The extract was tested for pyruvate and ethanol production from glucose. Pyruvic acid accumulated during the period of incubation and no ethanol was produced, Table 9. A second experiment with a fresh extract confirmed this. The extract was set up with phosphate buffer 2.2 μmoles, pyruvate 25 μmoles with succinic acid 5μmoles as hydrogen donor and the cofactors $MnCl_2 4H_2O$ 1.6 μmoles, $MgCl_2 6H_2O$ 2.4 μmoles, Cox 10 units and DPT 1 mg. per ml. There was no dissimilation of pyruvate.

A wet mass of cells, 5.4 g. was crushed in the Hughes' press and suspended in 30 ml. phosphate buffer containing 35 mg./l. nicotinamide. This crushed cell

Table 10.

Comparison of pyruvate and ethanol production from glucose (25 μmoles/ml.), glucose utilization and pyruvate dissimilation by whole cell and crushed cell suspensions and a cell-free extract obtained from crushed cells.

	Length of incubation (min.)	0	30	60	90	120
Whole cell suspension	Glucose utilized (μmoles/ml.)	0	4.5	6.0	11.0	-
	Pyruvate produced (μmoles/l.)	0	92	80	40	-
	Ethanol produced (μmoles/ml.)	0	4.5	11.5	4.6	-
Crushed cell suspension	Glucose utilized (μmoles/ml.)	0	3.15	6.0	11.0	11.0
	Pyruvate produced (μmoles/l.)	0	370	370	380	370
	Ethanol produced (μmoles/ml.)	0	2.8	2.9	2.9	2.8
	Pyruvate dissimilated (μmoles/ml.)	0	11.75	24.5	24.5	-
Extract from crushed cells	Glucose utilized (μmoles/ml.)	0	1.25	1.87	-	1.87
	Pyruvate produced (μmoles/l.)	0	0	0	0	0
	Ethanol produced (μmoles/ml.)	0	0	0	0	0
	Pyruvate dissimilated (μmoles/ml.)	0	0	2.35	3.0	2.5

Table 11.

The effect of inhibitors on pyruvate and ethanol production from glucose.

		Pyruvate production (umoles/l.)				Ethanol production (umoles/ml.)	
Length of Incubation (min.)		37	69	97	129	37	69
Substrate	Inhibitor						
Glucose	-	155	525	335	415	10.7	12.8
(25 umoles/ml.)	As_2O_3 (0.0025 M)	770	800	570	535	0	0
	NaF (0.04 M)	130	145	75	175	0	1.15
	KCN (0.05 M)	60	75	55	53	0	0

suspension and the extract obtained by centrifuging it, were tested for pyruvate and ethanol production from glucose, and for pyruvate dissimilation. The results were compared with those of a whole cell suspension (Table 10). The original cell suspension utilized glucose and pyruvate and produced ethanol from glucose but not from pyruvate. The crushed cell suspension also utilized glucose and pyruvate, but did not produce ethanol from either. Pyruvic acid accumulated during the glucose utilization. The extract was completely inactive.

The effect of inhibitors on pyruvate and ethanol production from glucose.

Tubes containing phosphate buffer 13 ml., glucose (0.166 M) 4 ml., cell suspension 2 ml. and inhibitor or water 1 ml. were incubated in an atmosphere of air at 37°. Samples were withdrawn at 37, 69, 97 and 128 min. and centrifuged. Pyruvate and ethanol estimations were carried out on the supernatants. Three inhibitors were used, arsenite (0.0025 M), sodium fluoride (0.04 M) and potassium cyanide (0.05 M). The results are given in Table 11. In the absence of inhibitors, both pyruvic acid and ethanol were produced from glucose. Arsenite inhibits pyruvate utilization: in the presence of arsenite there was accumulation of pyruvate and complete inhibition of ethanol production. Fluoride blocks the Embden Meyerhof pathway

Table 12.

Dissimilation of pyruvate by cell suspensions of *Escherichia coli* in the presence of different hydrogen donors and various cofactors.

	Pyruvate concentration (µmoles/ml.)					
Length of incubation (mins.)	0	15	30	45	60	90
Omissions from System A						
Cell suspension	24.5	-	-	-	-	24.6
Pyruvate	0.02	-	-	-	-	0.02
None	23.94	21.66	17.1	9.55	5.13	1.33
Succinic + cofactors	23.94	23.18	17.1	9.12	4.94	1.33
Additions to System B						
Na lactate	0.04	-	0.04	-	0.05	0.01
Na pyruvate + Na lactate	26.03	-	17.86	-	5.7	1.14
Na malate	0.05	-		-	0.05	0.03
Na pyruvate + Na lactate	26.03	-	17.86	-	3.04	1.14

System A. Phosphate buffer 1.3 µmoles, sodium pyruvate 25 µmoles, sodium succinate 5 µmoles, $MgSO_4.7H_2O$ 1.3 µmoles and cell suspension 10 mg. dry weight; Cofactors: CoA 10 units, glutathione 5 µmoles, DPT 1 mg., DPN 16 µg., DPNH 16 µg. and ATP .07 µmoles. per ml.

System B. Phosphate buffer 2.2 µmoles, cell suspension 10 mg. dry weight and $MgSO_4.7H_2O$ 1.0 µmoles per ml. Additions of sodium pyruvate 25 µmoles/ml., sodium lactate 5 µmoles/ml. and sodium malate 5 µmoles/ml. were made as indicated.

before the pyruvate stage. With the concentration of sodium fluoride used in this experiment, the inhibition was not complete and some pyruvate was produced, accompanied by a small amount of ethanol. Cyanide, which also inhibits pyruvate production, caused an even greater inhibition of pyruvate production and completely inhibited the production of ethanol.

Dissimilation of pyruvate by cell suspensions.

System A: Phosphate buffer 1.3 µmoles, sodium pyruvate 25 µmoles, sodium succinate 5 µmoles, $MgSO_4.7H_2O$ 1.3 µmoles, cell suspension 10 mg. dry weight; co-factors - CoA 10 units, glutathione 5 µmoles, TPP 1 µg., DPN 16 µg., TPNH 16 µg. and ATP 0.07 µmoles per ml. Tubes containing this complete system, and the system with the various omissions noted in Table 12, were incubated under an atmosphere of H_2 at 37°. Samples were withdrawn at 0, 15, 30, 45, 60, 90 min., centrifuged and pyruvate and ethanol estimations carried out on the supernatants. The dissimilation of the pyruvate was definitely due to the presence of cells. The addition of a hydrogen donor, succinate, and several cofactors did not affect the rate or products of pyruvate dissimilation. No ethanol could be detected under any of these conditions.

Lactic and malic acids were used as the hydrogen donors in system B. This contained phosphate buffer

2.2 μmoles, cell suspension 10 mg. dry weight and $MgSO_4.7H_2O$ 1.0 μmoles per ml. Additions of sodium pyruvate 25 μmoles, sodium lactate 5 μmoles and sodium malate 5 μmoles were made per ml. The tubes were incubated under the same conditions with similar results, i.e., no ethanol was produced although the pyruvate was rapidly dissimilated.

An attempt to obtain ethanol from pyruvate by priming the reaction with glucose was made. Tubes were set up as follows:

	Control	A	B	C
Phosphate buffer	3	3	3	3
Na pyruvate	-	3	2.4	1.8
Glucose	-	0.33	0.66	1.0
Water	3.7	0.37	0.64	0.9
$MgSO_4.7H_2O$ (10% W/V)	0.4	0.4	0.4	0.4
Na lactate	1.0	1.0	1.0	1.0
Cell suspension	2.0	2.0	2.0	2.0

Each tube contained sodium lactate 5 μmoles/ml. Each tube, except the control, contained the equivalent of 26 μmoles pyruvate/ml. made up as follows:-

A, 2 μmoles glucose (≡ 4μmoles pyruvate) + 22 μmoles pyruvate
B, 4 μmoles glucose (≡ 8 μmoles pyruvate) + 18 μmoles pyruvate
C, 6 μmoles glucose (≡ 12 μmoles pyruvate) + 14 μmoles pyruvate.

Table 13.

Dissimilation of pyruvate and production of ethanol in the presence of varying amounts of glucose. A 2 μmoles/ml., B 4 μmoles/ml. and C 6 μmoles/ml.

	Initial glucose concentration μmoles/ml.	Pyruvate Concentration (μmoles/ml.)				Ethanol produced (μmoles/ml.)
Length of incubation (min.)		0	30	60	90	60
Tube A	2	27.0	19.3	9.5	3.5	1.4
B	4	19.2	11.45	7.0	3.4	2.7
C	6	14.1	10.0	5.5	3.3	4.7

The tubes were incubated under an atmosphere of N_2 at 37° and samples withdrawn at 0, 30, 60, 90 min. and centrifuged. The results of the pyruvate and ethanol estimations on the supernatants are given in Table 13. The pyruvate was dissimilated. The ethanol produced, however, could be accounted for in each case as coming from the glucose present, and not from the pyruvate.

Dissimilation of pyruvate by cell-free extracts.

A crushed cell extract, and one prepared from alumina ground freeze-dried powder, would not dissimilate pyruvate in the system: phosphate buffer 1.5 μmoles, pyruvate 24.5 μmoles, succinate 5 μmoles, CoA 10 units, glutathione 5 μmoles, DPT 1 mg., $MgSO_4.7H_2O$ 1.3 μmoles, $MnSO_4.4H_2O$ 2.2 μmoles, DPN 16 μg., $DPNH_2$ 16 μg. and ATP 32 μg. per ml. The crushed cell extract was prepared from an active cell suspension and the crushed cell suspension, which was also tested (Table 10), was able to dissimilate pyruvate although it did not produce any ethanol either from glucose or pyruvate. The pyruvate splitting enzyme would thus appear to remain with the cell debris and not to be extracted by phosphate buffer, while some other enzyme between pyruvate and ethanol seems to be inactivated by the crushing. It is not alcohol dehydrogenase, although it must be remembered that this may not

function in the production of ethanol in the cell, because extracts prepared by both these methods contain an active alcohol dehydrogenase.

Coenzyme A and Ethanol Production

An extract from an acetone dried powder of Escherichia coli was treated with Dowex-1, in an attempt to establish that CoA is required in the production of ethanol. It had previously been observed that such extracts often had more activity for producing ethanol from α-glycerophosphate than from glucose (16 µmoles from 25 µmoles α-glycerophosphate, 4 µmoles from 25 µmoles glucose) and so both these substrates were used in this experiment. 40 ml. extract were prepared from 0.8 g. acetone dried powder. 15 ml. Dowex-1 was added to 30 ml. chilled extract, the pH adjusted to 7.2 and the mixture allowed to stand for 30 min. in an ice bath (Chantrenne and Lipmann 1950, Stadtman et al. 1951). The 'treated' extract, obtained after centrifuging off the Dowex-1, and the untreated extract were tested for ethanol production from glucose and α-glycerophosphate with and without the addition of CoA, and yeast extract. The system used was, glucose 25 µmoles, or α-glycerophosphate 25 µmoles, $MgCl_2.6H_2O$ 2.4 µmoles, $MnCl_2.4H_2O$ 1.6 µmoles, CoA (when added) 8 units and yeast extract (when added) 0.06%

Table 14.

Production of ethanol from glucose (25 μmoles/ml.) and α-glycerophosphate (25 μmoles/ml.) by a cell-free extract and a Dowex-1 treated cell-free extract of Escherichia coli with and without the addition of CoA and yeast extract.

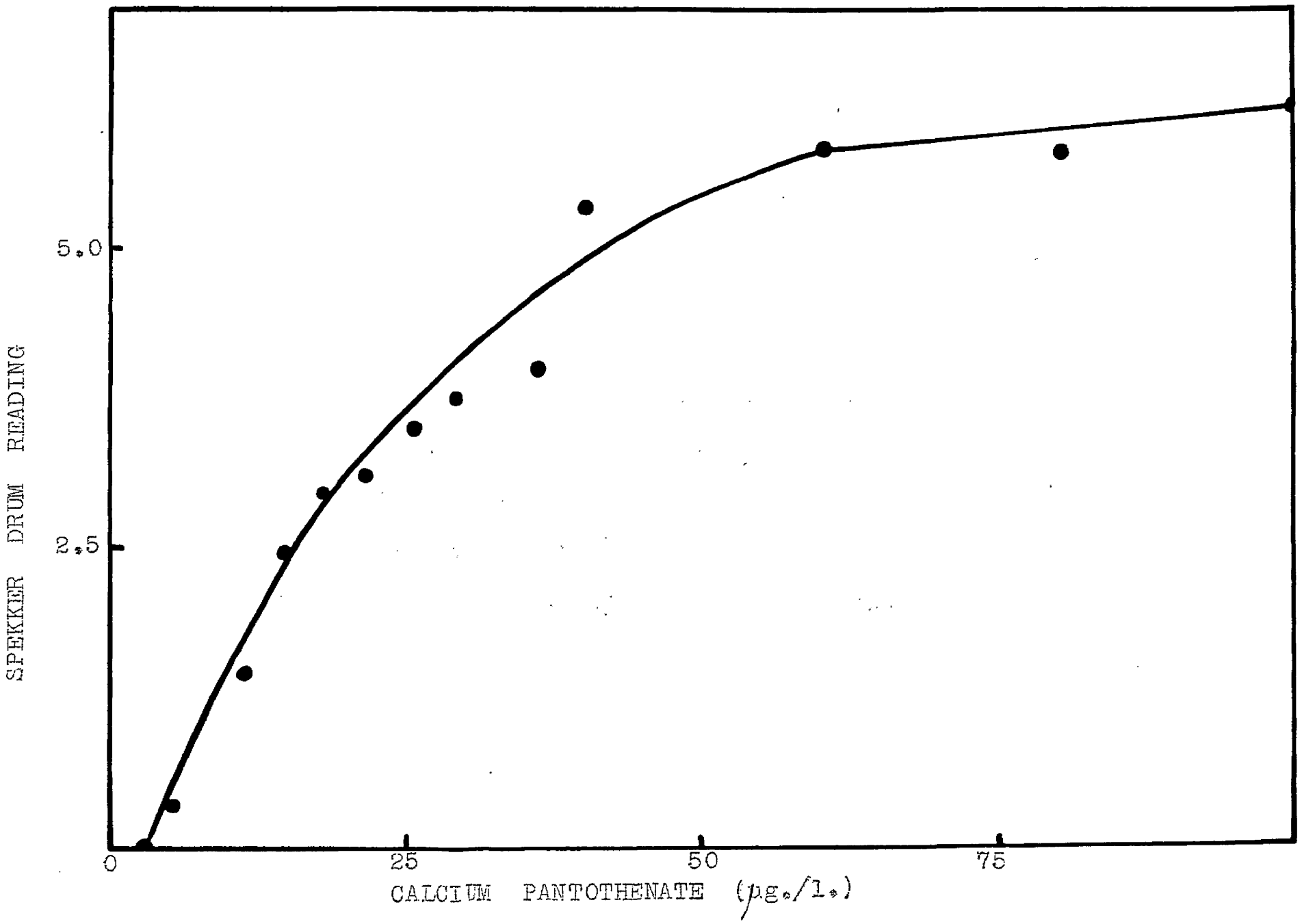
	Ethanol Production in 30 min. (μmoles/ml.) from	
	(a) Glucose	(b) α-Glycerophosphate
Untreated extract	4.75	7.5
Untreated extract + CoA	3.5	7.2
Untreated extract + yeast extract	3.5	7.3
'Treated' extract	3.5	1.1
'Treated' extract + CoA	3.8	6.3
'Treated' extract + yeast extract	4.2	4.6

per ml. The ethanol figures reported in Table 14 were those estimated after 30 min. under an atmosphere of N_2 at 37°. The results with glucose were not satisfactory, but the extract had little activity for producing ethanol from glucose. With α-glycerophosphate where there was almost twice the activity for ethanol production, the treatment with Dowex-1 almost completely removed this activity. Yeast extract partially restored the activity, while there was almost complete restoration by CoA.

Experiments with a pantothenate-requiring mutant of Escherichia coli

The object of these experiments was to investigate whether CoA was necessary for ethanol production. The organism used was Escherichia coli mutant 99-1, which requires pantothenate and will not respond to any of its precursors. This was kindly supplied by Dr. Bernard D. Davis, of the United States Public Health Service, Tuberculosis Research Laboratory, New York. Davis and Mingioli (1950) gave as the basal minimal medium for the growth of the mutant one containing KH_2PO_4 7.0 g., K_2HPO_4 3.0 g., sodium citrate 0.5 g., $MgSO_4.7H_2O$ 0.1 g., $(NH_4)_2SO_4$ 1.0 g. and glucose 2.0 g. per litre, adjusted to pH 7.0, and found that maximum growth response was obtained with ca. 200 mµg. per 10 ml. pantothenate. This medium

Fig.5. Growth of the pantothenate-requiring mutant 90-1 of Escherichia coli, expressed as Spekker drum reading, in relation to the concentration of pantothenate in glucose-ammonium salt medium.


SPEKKER DRUM READING
5.0
2.5
0
25
50
75
CALCIUM PANTOTHENATE (µg./l.)

was used to maintain the stock culture. The mutant also grew on glucose-ammonium salt medium, pH 7.1, when 500 mg. pantothenate were added to 25 ml. of this medium contained in a 6 x 1 in. Pyrex tube.

The growth response of the Esch. coli mutant to pantothenate was studied. Tubes containing 25 ml. glucose-ammonium salt medium were set up with increasing concentrations of pantothenate, ranging from 2.91 to 400 µg. per litre. These were inoculated with 1 loop of glucose-ammonium limiting pantothenate grown cells, and incubated at 37°. When the cultures were fully grown, 1 ml. of each was withdrawn, killed with one drop of formalin and the density of the cultures measured in the standard way using a Spekker photoelectric absorptiometer with Ilford filters neutral H500 and blue 082. In the absence of a calibration curve relating optical density to the number of cells for this particular organism, the Spekker drum readings were plotted against the concentrations of pantothenate, Fig.5. The growth response was dependent upon the pantothenate up to a concentration of 50 µg. per litre. Three concentrations of pantothenate were chosen for the next experiment: 100 µg/l. to give cells grown in excess pantothenate, and 40 µg/l. and 15 µg/l. to give cells grown in limiting concentrations of pantothenate.

Table 15.

Analysis of the supernatants from cultures of Escherichia coli mutant grown in glucose-ammonium salt medium containing different amounts of pantothenic acid.

Pantothenic Acid in Medium	n_s	Pyruvate Concentration	Ethanol Concentration
(µg./l.)	(10^6 cells/ml.)	(µmoles/10^6 cells/l.)	
15	170	5.15	25
40	575	7.06	0.25
100	600	5.68	0.7

Table 16

Production of pyruvate from glucose (25 µmoles/ml.) by cell suspensions of Escherichia coli mutant grown in glucose ammonium salt media containing different amounts of pantothenic acid

Pantothenic Acid in Media (µg./l.)	Pyruvate Production by Cell Suspensions (µmoles/mg. dry wt./ml.)	
	60 min.	120 min.
15	132	97
40	46	38
100	2.1	25

Fig.6. Production of ethanol from glucose (25 μmoles/ml.) by non-proliferating cell suspensions of *Escherichia coli* mutant 99-1 grown in glucose-ammonium salt media containing (a) limiting concentrations of pantothenate, ○ 15 μg./l. and ● 40 μg./l., and (b) a concentration in excess of growth requirements of pantothenate □ 100 μg./l.

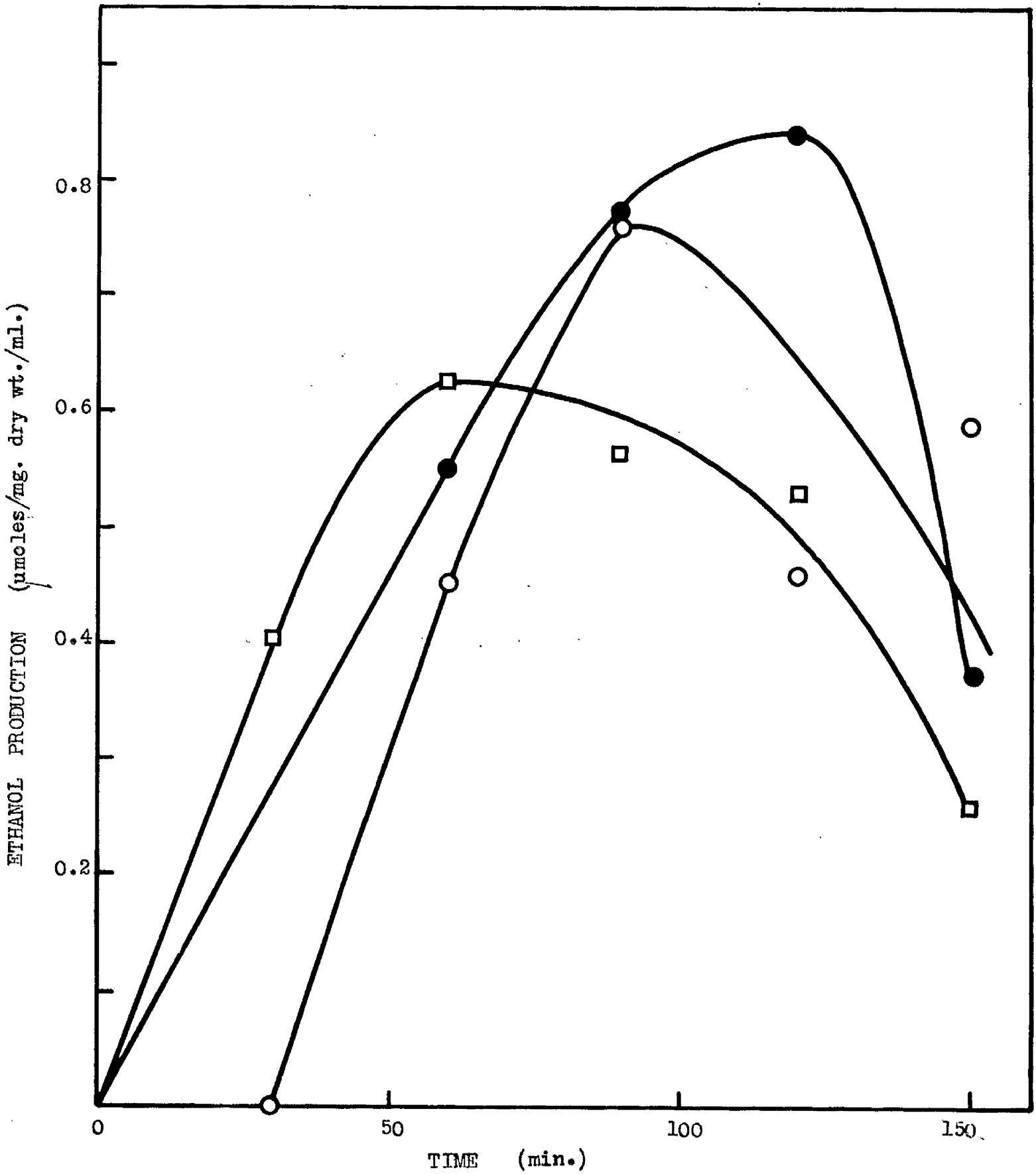
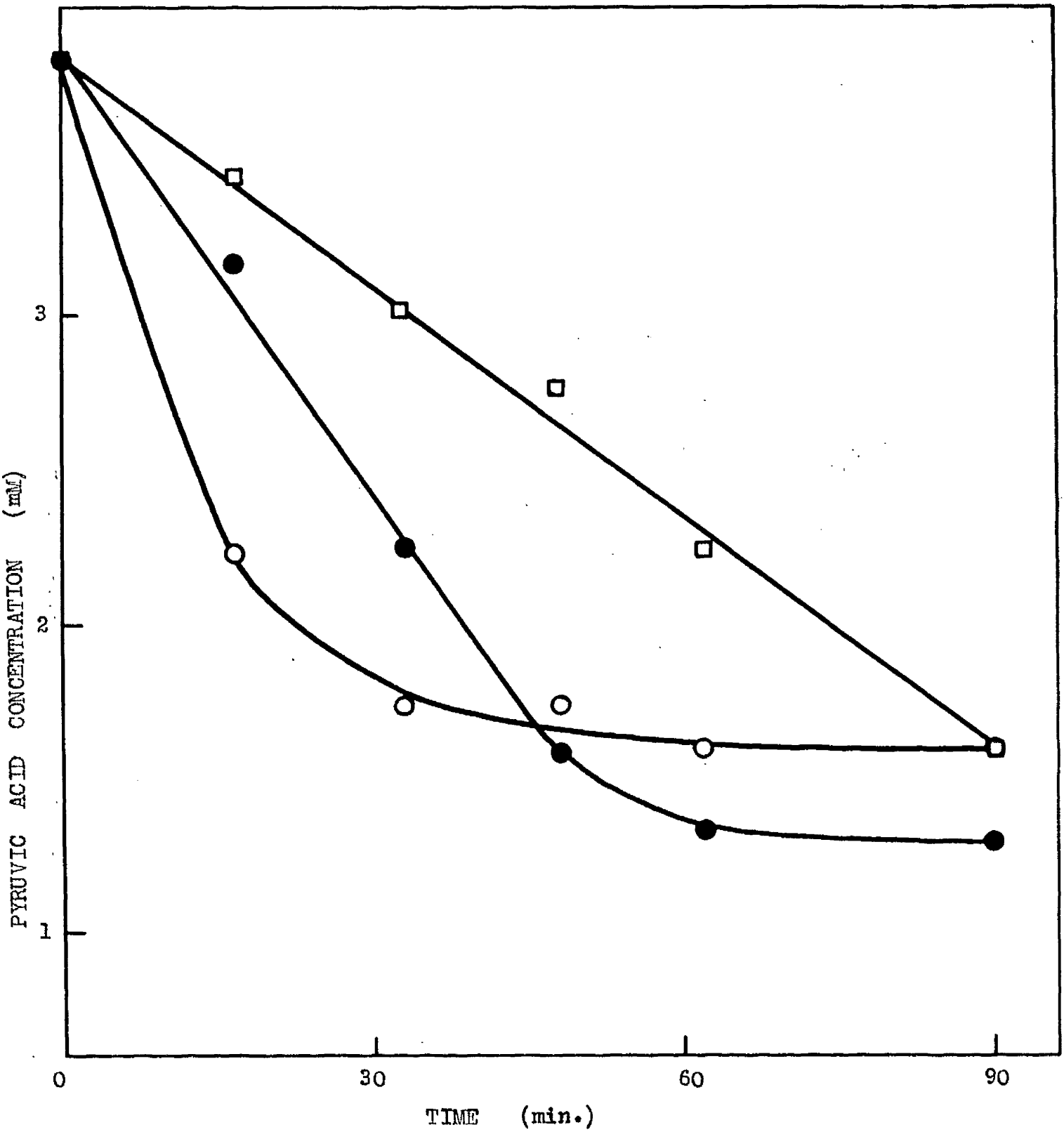

0.8
0.6
0.4
0.2
ETHANOL PRODUCTION (µmoles/mg. dry wt./ml.)
0
50
100
150
TIME (min.)

Fig.7. Dissimilation of pyruvic acid by non-proliferating cell suspensions of Escherichia coli mutant 99-1 grown in glucose-ammonium salt media containing (a) limiting concentrations of pantothenate, □ 15 µg./l. and ● 40 µg./l., and (b) an excess of pantothenate, ○ 100 µg./l.


PYRUVIC ACID CONCENTRATION (mM)
3
2
1
0
30
60
90
TIME (min.)

3 litres of glucose-ammonium salt medium containing 15 µg/l. pantothenate were set up in a 5 litre flask. 2 separate litres of the same medium containing 40 µg/l. and 100 µg/l. pantothenate respectively were set up in 2 litre flasks. These were all inoculated with the mutant and incubated at 37°. The cells were harvested, washed and suspended in 18 ml. phosphate buffer. The growth supernatant was analysed for pyruvate and ethanol, and the stationary populations measured, Table 15. The production of pyruvate and ethanol from glucose by the cell suspensions was studied under the usual conditions for the ethanol activity test. The pyruvate produced from glucose by the three suspensions is given in Table 16. The ethanol results are shown in Fig.6. Dissimilation of pyruvate by the cell suspensions was studied. 5 ml. of each suspension, adjusted to give 3 mg. dry weight/ml., were set up in the tubes containing 10 ml. phosphate buffer and 10 ml. pyruvate solution to give a final concentration of ca. 3,500 µmoles. Suitable controls were set up. The tubes were incubated at 37° and samples withdrawn over a period of 95 mins. The results are given in Fig.7.

The concentration of pyruvate found in any time is a measure of the amount which has been produced but

not utilized, i.e., a reflexion of the balance between production and utilization. Ethanol concentration is known to reach a maximum and then decline (Fig.4) in washed cell suspensions and, therefore, with a washed cell suspension at least the ethanol concentration represents the balance between production and utilization. The fact that ethanol concentration in the growth supernatant decreased with increasing concentrations of pantothenate may mean that the utilization of ethanol is faster in those cultures provided with excess pantothenate. Pyruvate accumulated in those suspensions which had grown with limiting concentrations of pantothenate and which would contain, as a consequence, minimal concentrations of CoA. This would determine pyruvate utilization. The overall dissimilation of pyruvate was the same in each suspension, but the rates were considerably affected. The greater the deficiency of CoA, the slower was the dissimilation. With ethanol production a similar picture was found. The ethanol concentration in each case reached a maximum and then declined. This maximum value was very similar in each case, but the greater the pantothenate concentration in the growth medium, the sooner that maximum was reached. The slightly lower figures in the suspension from the culture provided with excess pantothenate, while probably of no great significance, could presumably have been caused

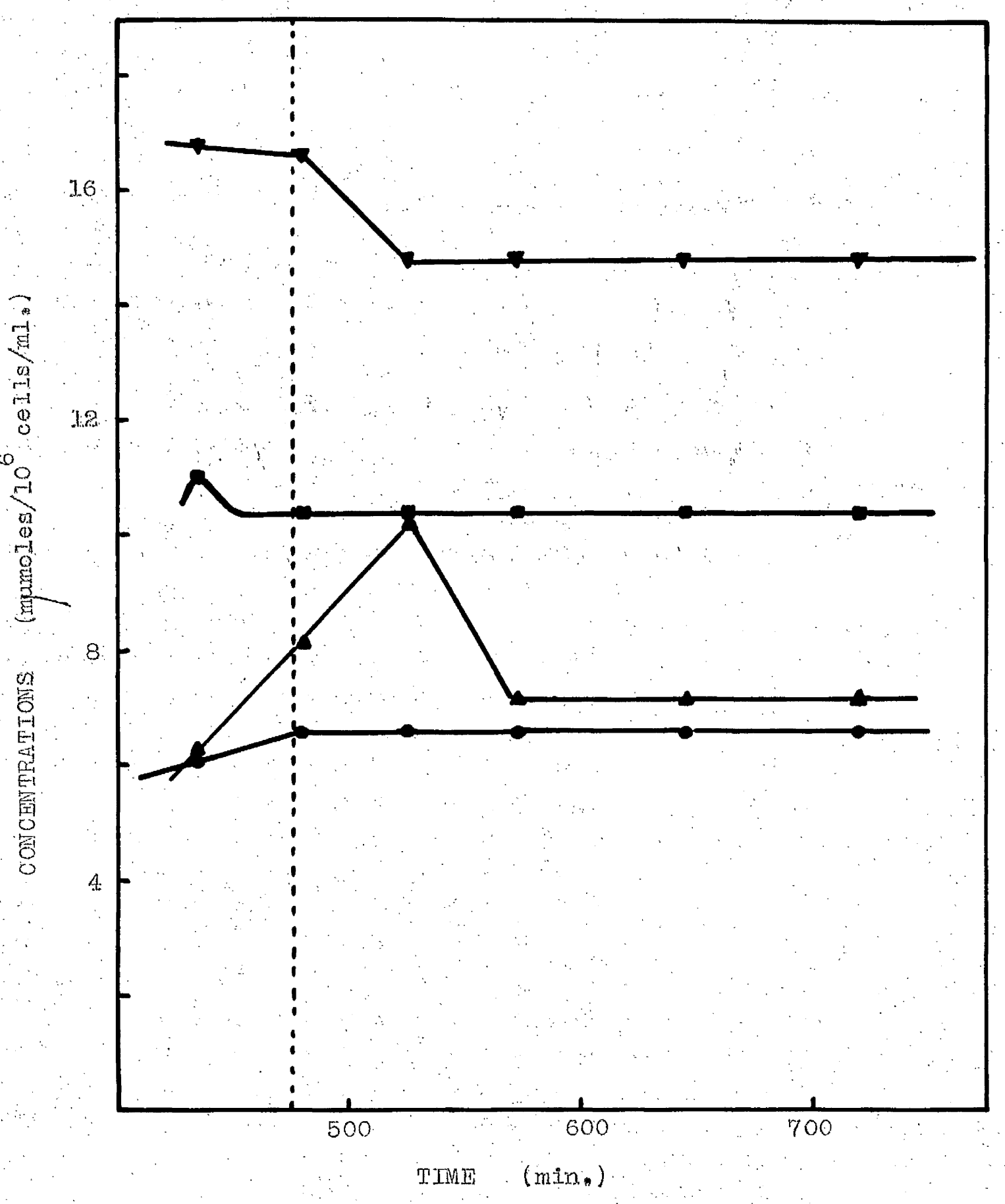
by a higher ethanol utilization by this suspension.

Products arising from the growth of Escherichia coli in a limiting concentration of glucose.

During previous work (Foster, 1952), the relationship of stationary population of Esch. coli to the glucose concentration of the medium was studied. It was found that when all other nutrients of glucose-ammonium salt medium were present in excess of growth requirements, growth was proportional to the glucose concentration up to a value of 1.25×10^{-2}M. If Esch. coli, therefore, were grown on a medium containing 9×10^{-3}M concentration of glucose, then when the stationary phase was reached all the glucose would be utilized. The object of this experiment was to study the balance of products which arose from limiting glucose in order to investigate any relationship between the ethanol and acetic acid yields.

500 ml. medium, containing KH_2PO_4 2.7 g., $(NH_4)_2SO_4$ 0.6 g., $MgSO_4.7H_2O$ 0.2 g. and glucose 0.81 g. and adjusted to ph 7.45, in a 1 l. Erlenmeyer flask were inoculated with 2 ml. of a glucose-ammonium salt medium grown culture of Esch. coli. Growth was followed and samples were withdrawn during growth and well into the stationary phase for the estimation of glucose utilized and ethanol, total volatile acids, acetic acid, lactic

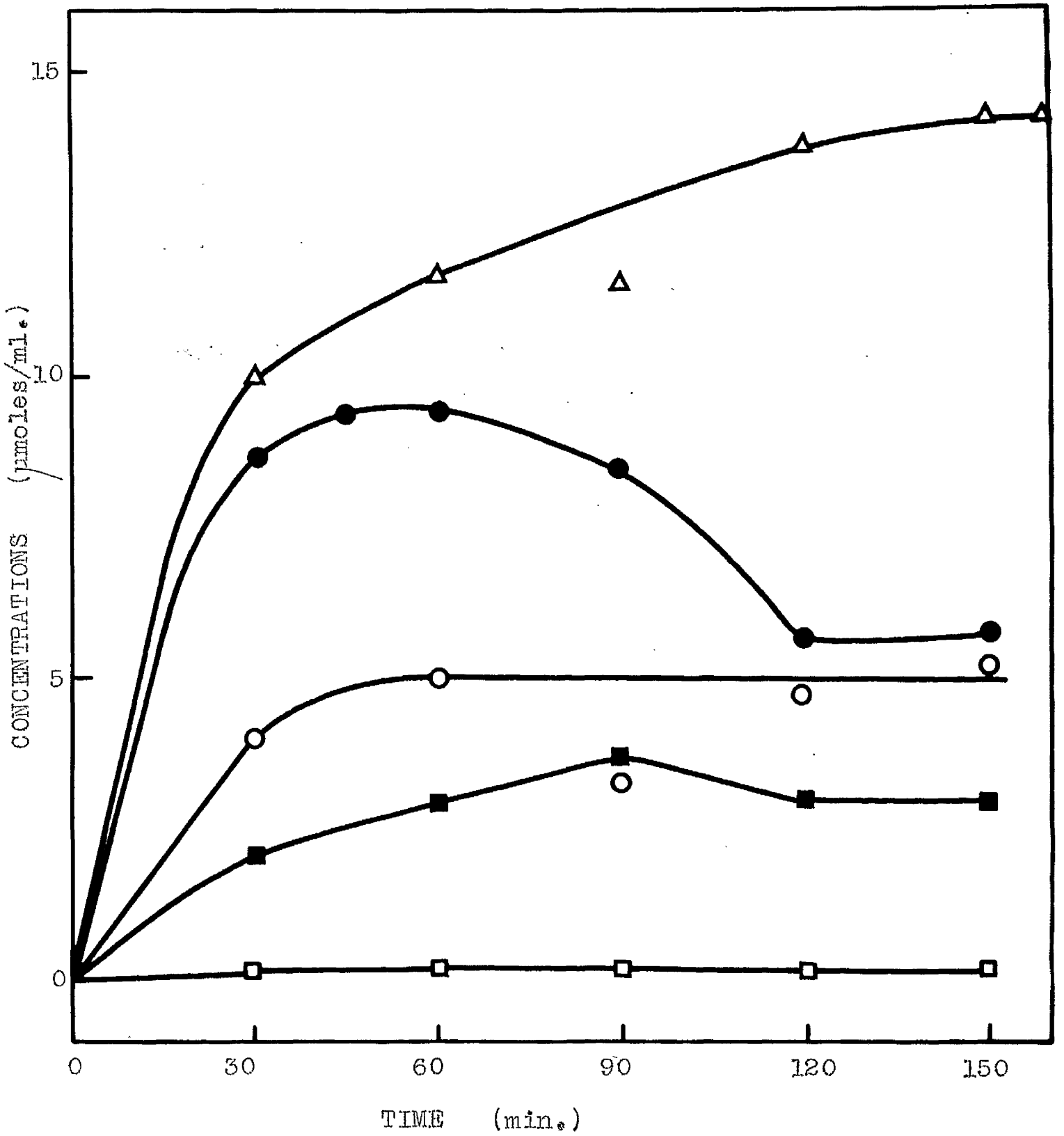
Fig.8. Balance of glucose utilized ■, and the ● acetic acid, ▼ total volatile acids, and ▲ ethanol produced in a culture of Escherichia coli after the stationary phase has been reached with a limiting concentration of glucose in glucose-ammonium salt medium, pH 7.45. Dotted line indicates the onset of the stationary phase.


16
12
8
4
CONCENTRATIONS (mμmoles/10^6 cells/ml.)
500
600
700
TIME (min.)

acid and total keto acids produced. Estimation of the glucose present in the medium showed that at the onset of stationary phase, 475 min. after inoculation and with a cell population of $1,185 \times 10^6$ cells/ml., the glucose was exhausted. The final pH value was 6.35. 50 min. after the onset of stationary phase, 9.75 µmoles/ml. glucose had been utilized, and 9.60 µmoles of ethanol had been produced. The acetic acid produced was 6.67 µmoles/ml., the formic acid 0.1 µmoles/ml. and lactic acid 0.2 µmoles/ml. No keto acids were detected. The products all remained at this level, except ethanol. In another 50 min. the ethanol concentration had fallen to the level of 7.21 µmoles/ml. giving an almost 1:1 ratio with the acetic acid. There was no change in the acetic acid concentration when the ethanol decreased. As reported in the growth experiments, at this pH the monocarboxylic acids do not account for the total acids produced as calculated from the pH change. The total acid produced was 24.3 µmoles/ml., giving 2.5 µmoles acid per µmole glucose. The other acid factors did not vary when the ethanol concentration decreased. A repeat experiment confirmed this drop in ethanol concentration while all the other products remained stable. The results are given in Fig.8, and from those obtained at 575 min. the following equation can be written:

Fig.9. A comparison of the rate and amount of production of some of the products of the fermentation of glucose by a non-proliferating cell suspension of Escherichia coli.

△ Glucose utilized; ◪ total keto acids; ■ lactic acid; O acetic acid; and ● ethanol.


15
10
5
0
CONCENTRATIONS (μmoles/ml.)
0
30
60
90
120
150
TIME (min.)

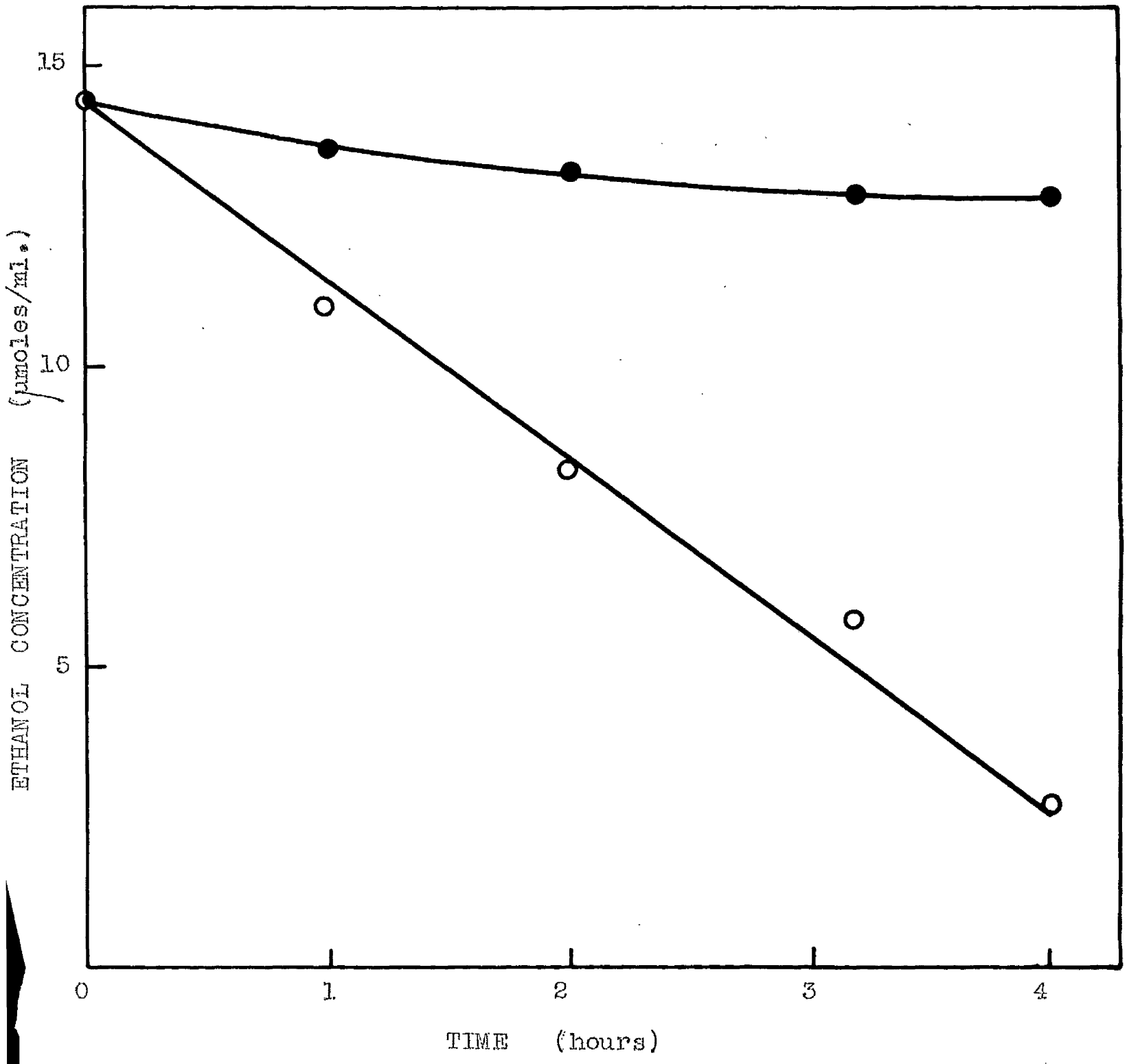
Glucose →	Ethanol	+ Acetic	+ Formic	+ Lactic	+ Other Acids
Moles: 1	0.74	0.69	0.83	0.02	1.02.

The oxidation of ethanol under anaerobic conditions.

A washed cell suspension of Esch. coli was set up as for the normal alcohol test, using a total of 150 ml. liquid in 8 x 2 in. tubes. Samples (25 ml.) were withdrawn at 0, 30, 60, 90, 120 and 150 min. and glucose, ethanol, acetic acid, lactic acid, total keto acid and acetyl phosphate estimations were carried out on the supernatants, in order to investigate whether any variation of the other fermentation products occurred when the ethanol concentration declined from its maximum. No acetyl phosphate was detected. The concentration of total keto acid, which was found to be made up of about half pyruvate and half α-ketoglutarate, never exceeded 0.3 µmoles/ml. There was no increase in the concentration of acetic, lactic or keto acids to correspond with the decrease in ethanol concentration, Fig.9.

The disappearance of ethanol from an anaerobic system was investigated. The system contained phosphate buffer, 2.2 µmoles, ethanol 15 µmoles, and cell suspension 10.2 mg. dry weight per ml. in a total of 1 ml. In the control the cell suspension was replaced by water. The concentration of ethanol in the control tube decreased

Fig.1. Disappearance of ethanol ● from a control tube containing ethanol and phosphate buffer, ○ in the presence of a non-proliferating cell suspension of Escherichia coli, under an atmosphere of nitrogen.

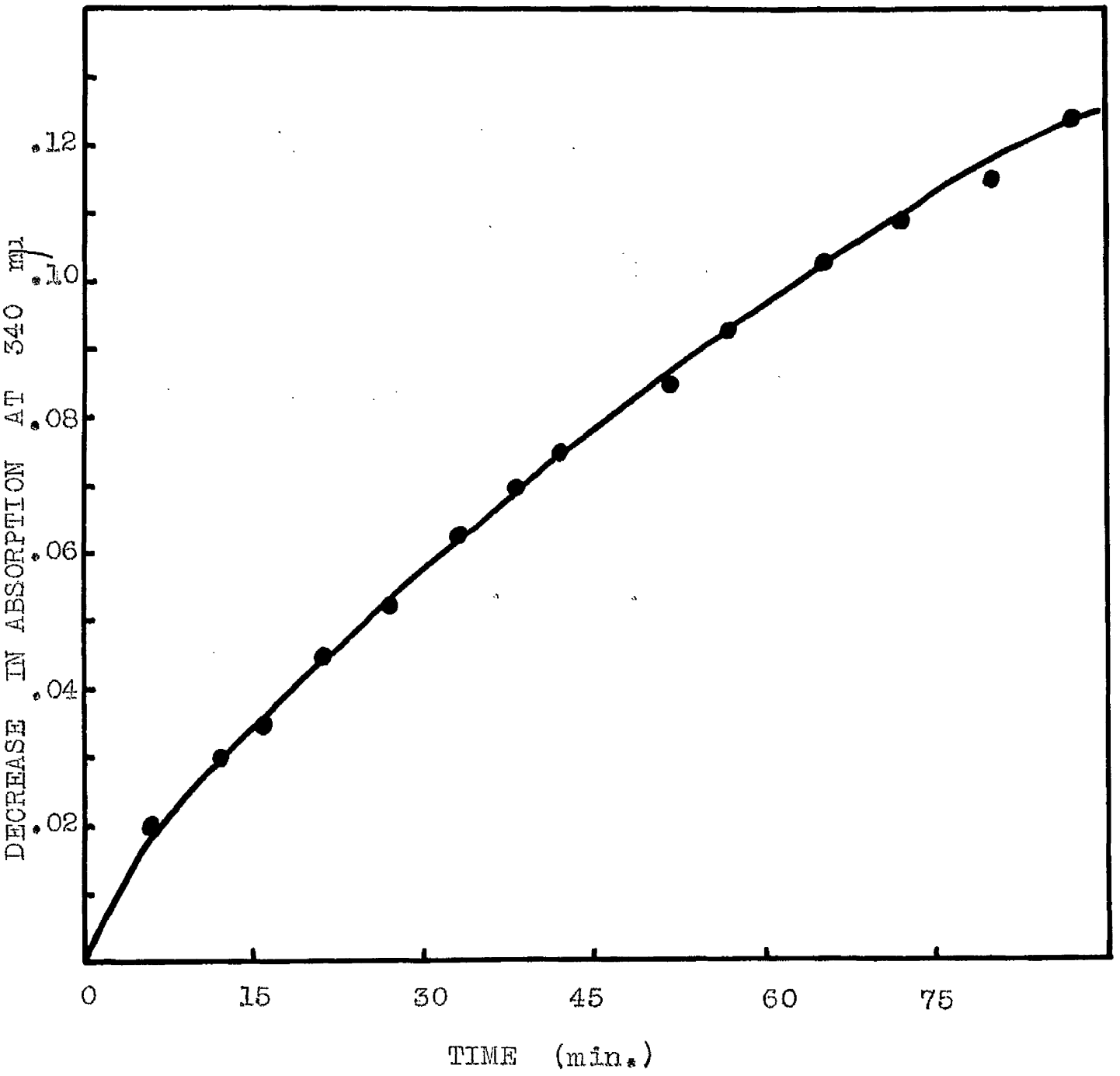

15
10
5
0
1
2
3
4
ETHANOL CONCENTRATION (μmoles/ml.)
TIME (hours)

steadily from 14.93 µmoles/ml. at the start of the experiment to 12.71 µmoles/ml. after 3 hours: a fall of 0.76 µmole/ml. per hour. When cells were present the ethanol concentration decreased steadily from 14.63 µmoles/ml. to 2.76 µmoles/ml. after 4 hours: a drop of 2.97 µmoles/ml. per hour (Fig.10). If fumarate (15 µmoles/ml.) was added to the system, the rate of ethanol disappearance was unaffected in the control, but was almost doubled in the presence of cells. Thus it would appear that the cells oxidize ethanol under these anaerobic conditions. The fate of this ethanol is unknown. There is definitely no increase in the concentration of acetic, lactic, formic or keto acids to account for it (Figs.8 and 9).

Alcohol dehydrogenase.

1 g. of freeze dried powder was ground with 2 g. alumina in a chilled agate pestle and mortar, and extracted with 20 ml. chilled phosphate buffer. The extract was saturated with $(NH_4)_2SO_4$ and the precipitate redissolved in water to give protein solution A. Alcohol dehydrogenase activity was assayed in a system containing phosphate buffer 6.6 µmoles, acetaldehyde 50 µmoles, reduced DPN ($DPNH_2$) 0.1 µmole, CoA 36 units, glutathione 5 µmoles, $MgSO_4.7H_2O$ 3.12 µmoles and extract 24 mg. protein per 3 ml. The phosphate buffer and

Fig.11. The decrease in absorption at 340 mμ due to the oxidation of $DPNH_2$ by acetaldehyde in the presence of protein solution A. The system contained phosphate buffer 6.6 μmoles, acetaldehyde 50 μmoles, $DPNH_2$ 0.1 μmole, CoA 36 units, glutathione 5 μmoles, $MgSO_4.7H_2O$ 3.12 μmoles and extract 24 mg. protein per 3 ml. The readings for a control cuvette have been subtracted.


DECREASE IN ABSORPTION AT 340 mμ
.12
.10
.08
.06
.04
.02
0
15
30
45
60
75
TIME (min.)

$MgSO_4.7H_2O$ solution were at room temperature. The other solutions were kept in an ice-bath. With the exception of the $DPNH_2$, all the components of the system were added to a cuvette (1 cm.). A control was prepared omitting the acetaldehyde. The absorption at 340 mμ was read on the Unicam S.P. 500 spectrophotometer. $DPNH_2$ was added at zero time, and readings taken from 1 min. onwards. The increase in absorption at 340 mμ due to the $DPNH_2$ at zero time was obtained by extrapolation of the curve back to zero time. The total volume in the cuvette was 3 ml. Fig.11 shows the decrease in absorption in the test cuvette after subtraction of the decrease in absorption of the control, which represents the change in absorption due to the oxidation of $DPNH_2$ by acetaldehyde and the extract.

5 g. wet weight of cells were crushed at -23° and extracted with 25 ml. phosphate buffer. The total protein was precipitated by full saturation with 20 g. $(NH_4)_2SO_4$ and redissolved in 25 ml. distilled water yielding protein solution C. This extract showed a similar activity to protein solution A. As this method of preparing extracts was much less time consuming than the previous one and resulted in an equally active preparation, it was adopted as the standard method. This extract showed a considerable decrease in absorption in the control

Table 17.

Fractionation of Protein Solution C.

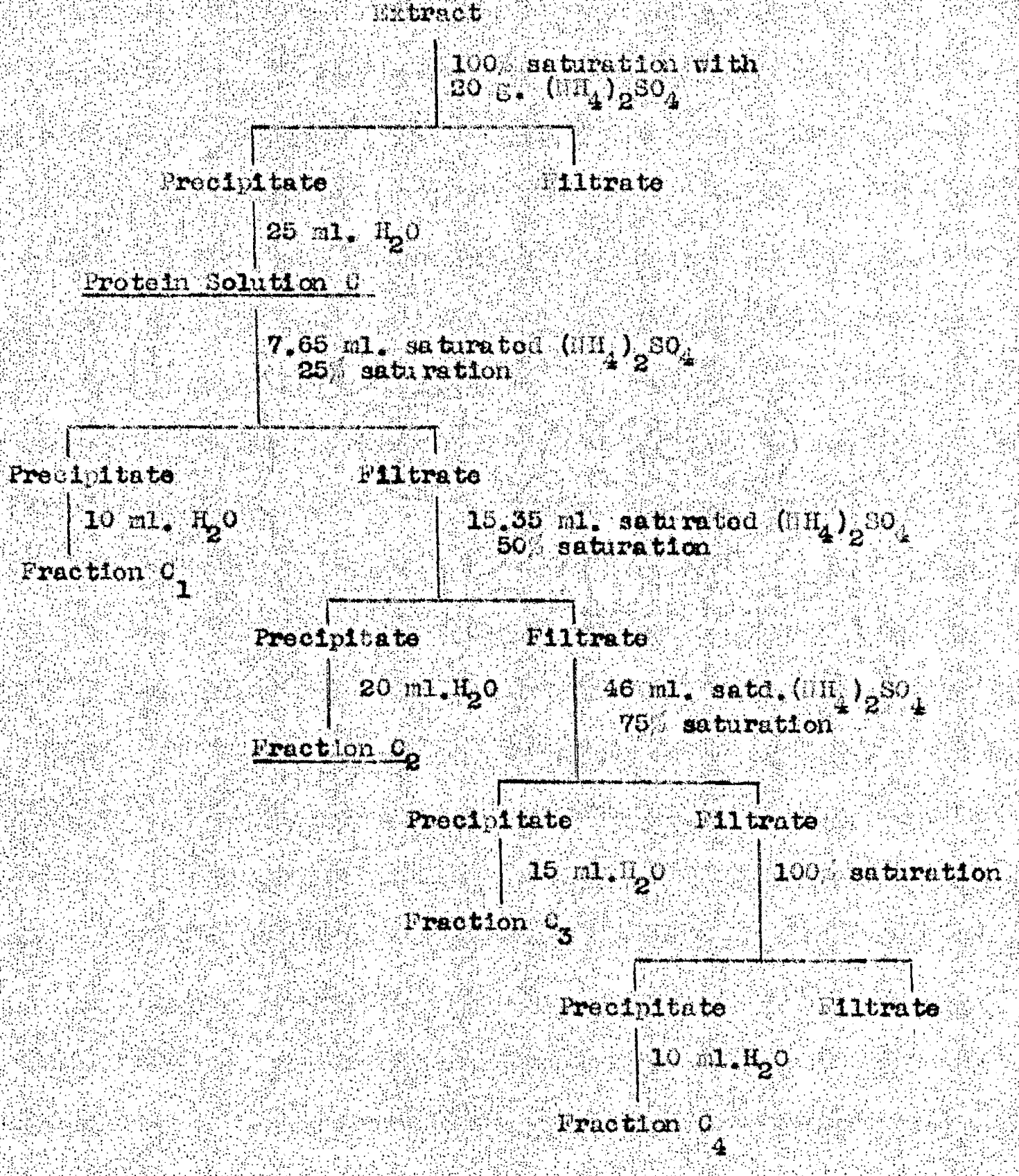
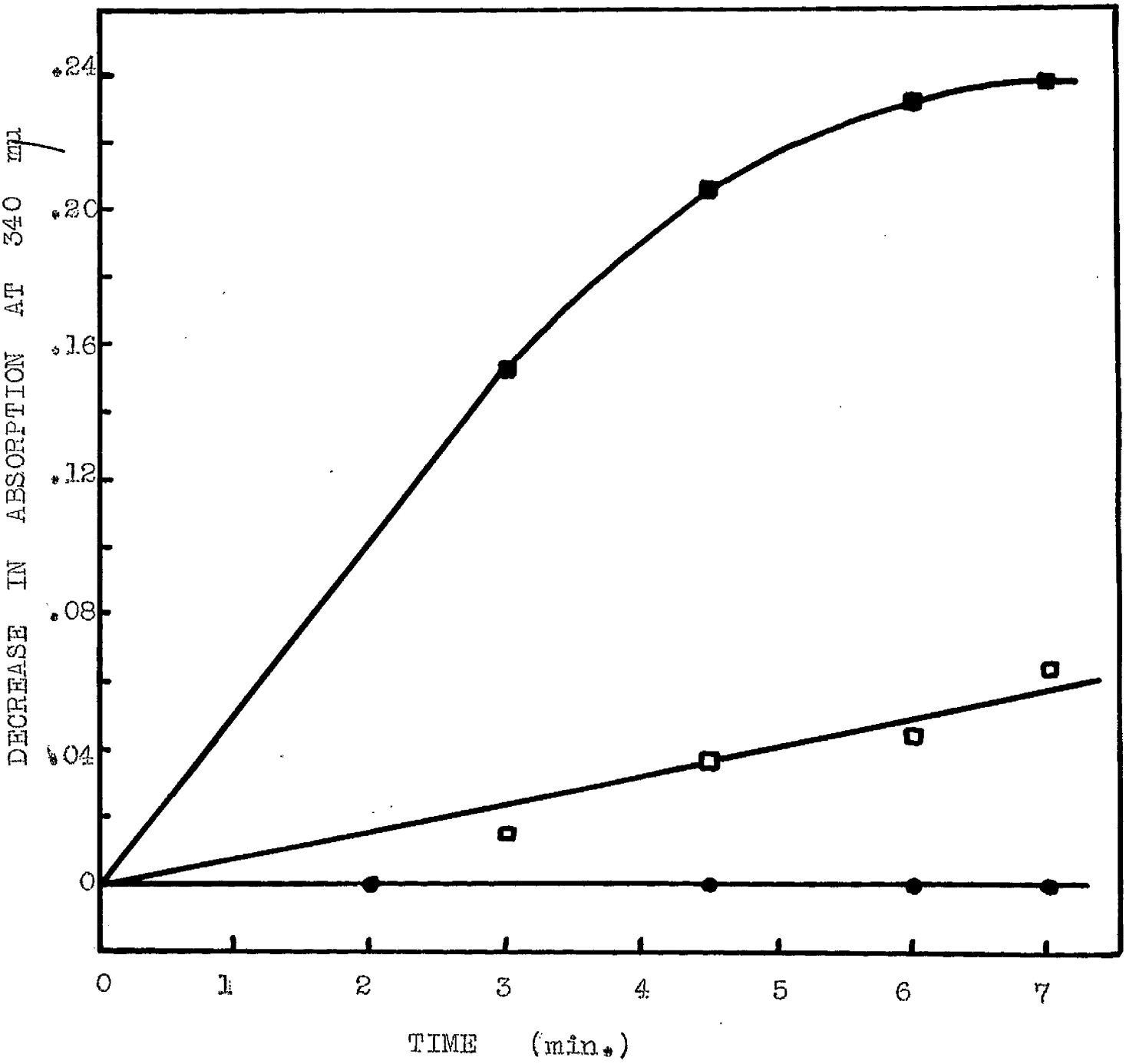


Fig.12. The decrease in absorption at 340 mμ due to the oxidation of $DPNH_2$ ■ in a system containing phosphate buffer 6.6 μmoles, $MgSO_4.7H_2O$ 3.12 μmoles, $DPNH_2$ 0.1 μmole, acetaldehyde 10 μmoles and fraction O_2 1.0 ml. per 3 ml. ● represents two controls, each giving identical readings, one with acetaldehyde and extract omitted, the other with extract omitted, and □ represents a control with acetaldehyde omitted.


DECREASE IN ABSORPTION AT 340 mμ
.24
.20
.16
.12
.08
.04
0
0
1
2
3
4
5
6
7
TIME (min.)

ouvothe. Two reasons were suggested for this: the oxidation of the $DPNH_2$ by the atmosphere, or the presence of other enzymes in the extract capable of oxidizing the $DPNH_2$. The extract was fractionated as shown in Table 17. CoA and glutathione were found to be unnecessary for activity and, therefore, were omitted from the system. The fractions were tested in a system containing phosphate buffer 6.6 µmoles, $MgSO_4.7H_2O$ 3.12 µmoles, $DPNH_2$ 0.1 µmole, acetaldehyde 10 µmoles and extract 1.0 ml. per 3 ml. Three controls were set up. In control (a) both extract and acetaldehyde were omitted: in control (b) the extract was omitted and in control (c) acetaldehyde was omitted.

The active fraction (C_2) was that which precipitated at 50% saturation with $(NH_4)_2SO_4$ and which contained 25.4 mg. protein per ml. As Fig.12 shows this still displayed some decrease in absorption at 340 mu in the absence of added substrate. The $DPNH_2$ remained stable over the period of the experiment, i.e., over 40 minutes. Acetaldehyde alone did not oxidize the $DPNH_2$. The complete system oxidized $DPNH_2$ in 7 min., while the extract alone had not completely oxidized it in 40 min. This activity shown by the extract alone could be reduced in two ways. If fraction C_2 was precipitated directly from the original extract, there was high control activity.

Fig.13. The effect of storing enzyme solution in the deep freeze cabinet for 2 days. The system contained phosphate buffer 6.6 μmoles, $MgSO_4.7H_2O$ 3.12 μmoles, $DPNH_2$ 0.1 μmole acetaldehyde 10 μmoles and extract 1.0 ml. per 3 ml. ■ Control tube with acetaldehyde omitted; ● complete system; (1) fresh extract; (2) stored extract. The arrows indicate maximum possible decrease, T for ●, C for ■, and B for both ● and ■.

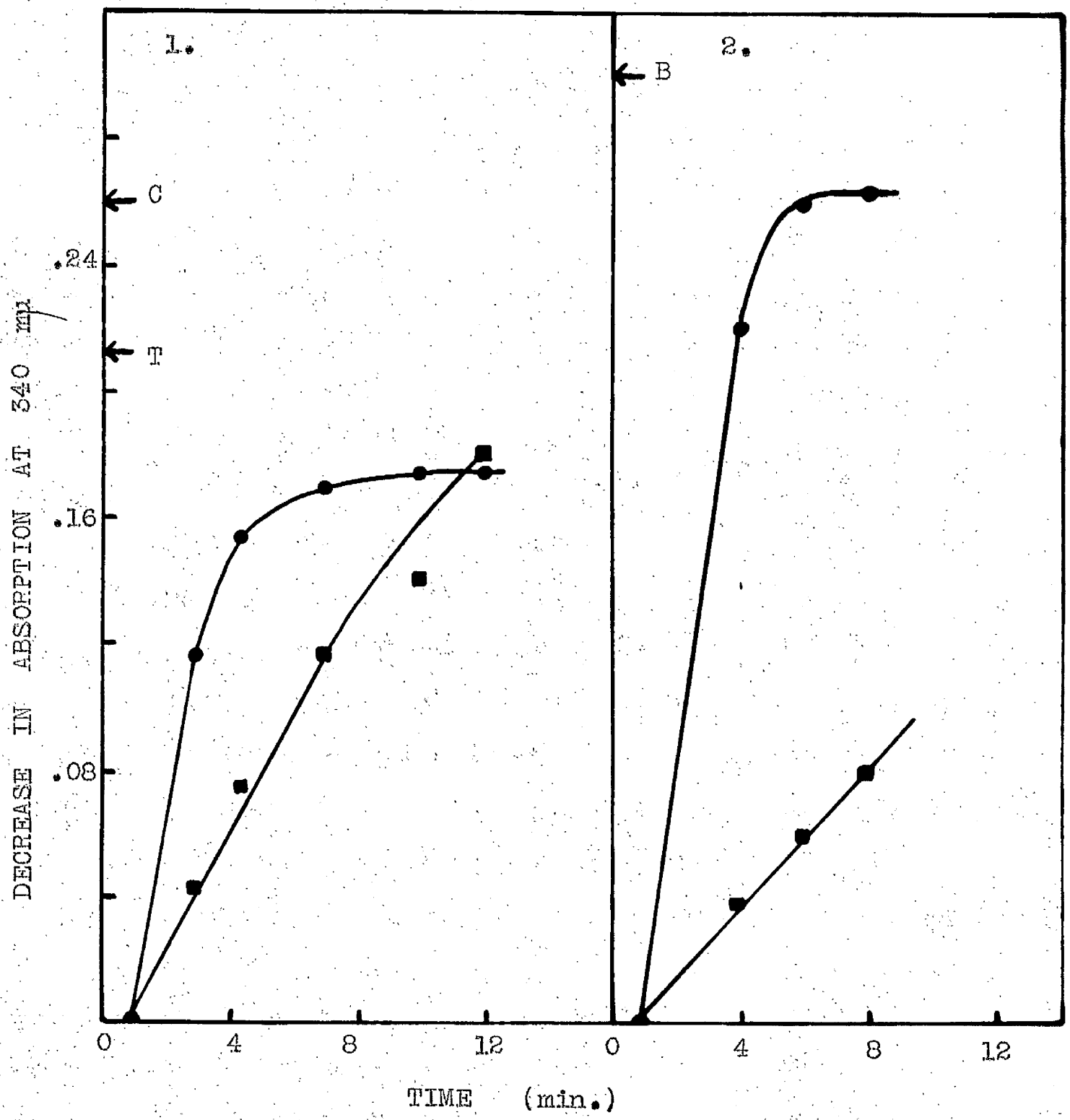
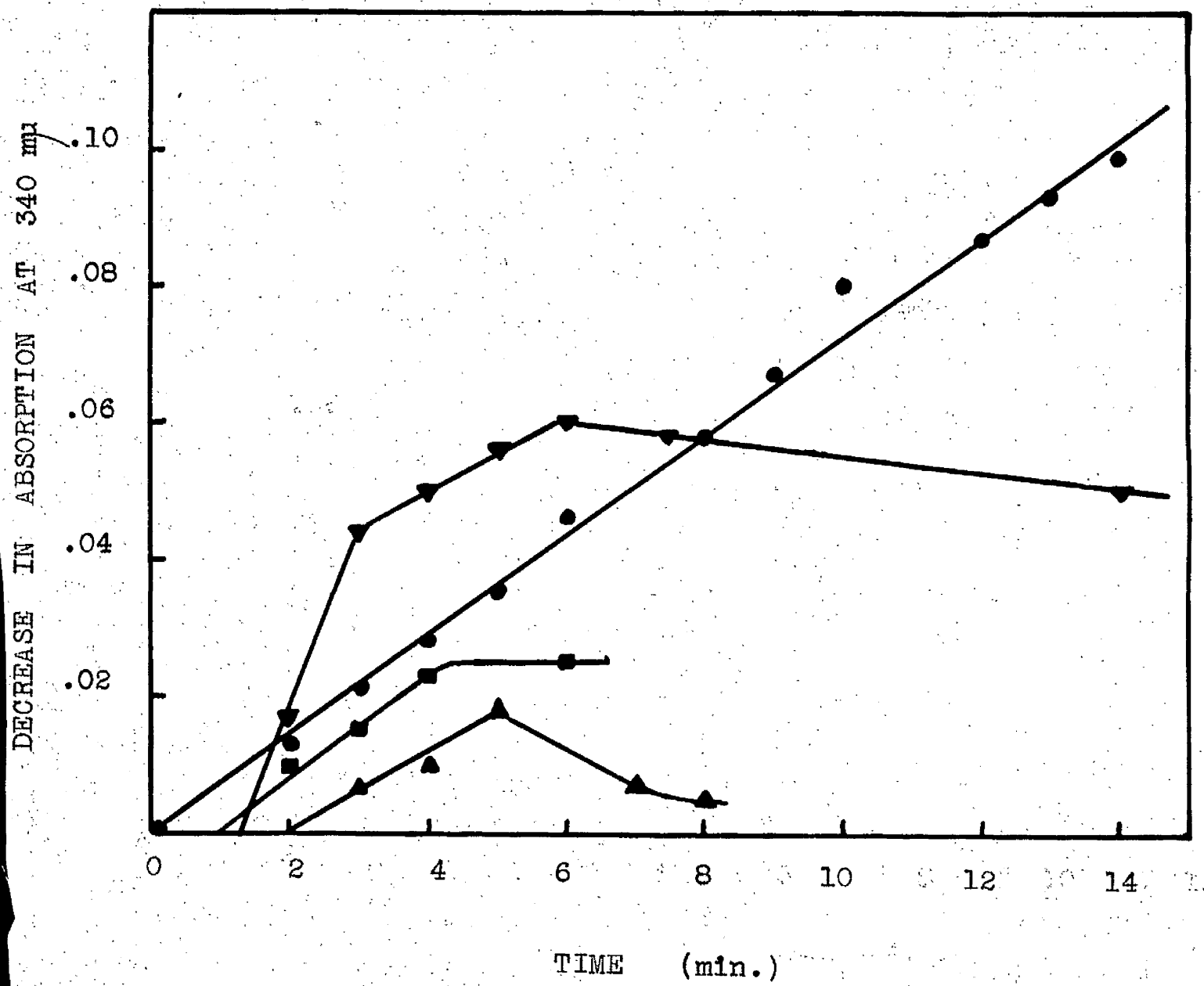

1.
2.
B
C
T
.24
.16
.08
DECREASE IN ABSORPTION AT 340 mμ
0
4
8
12
0
4
8
12
TIME (min.)

Fig.14. The effect of pH on the activity of fraction O_2 to reduce acetaldehyde in the system phosphate buffer of appropriate pH 6.6 µmoles, $MgSO_4.7H_2O$ 3.12 µmoles, DPN H_2 0.1 µmole, acetaldehyde 10 µmoles and extract 1.0 ml. per 3 ml. △ pH 8.5, □ pH 7.5, ● pH 6.5 and ○ pH 5.5.

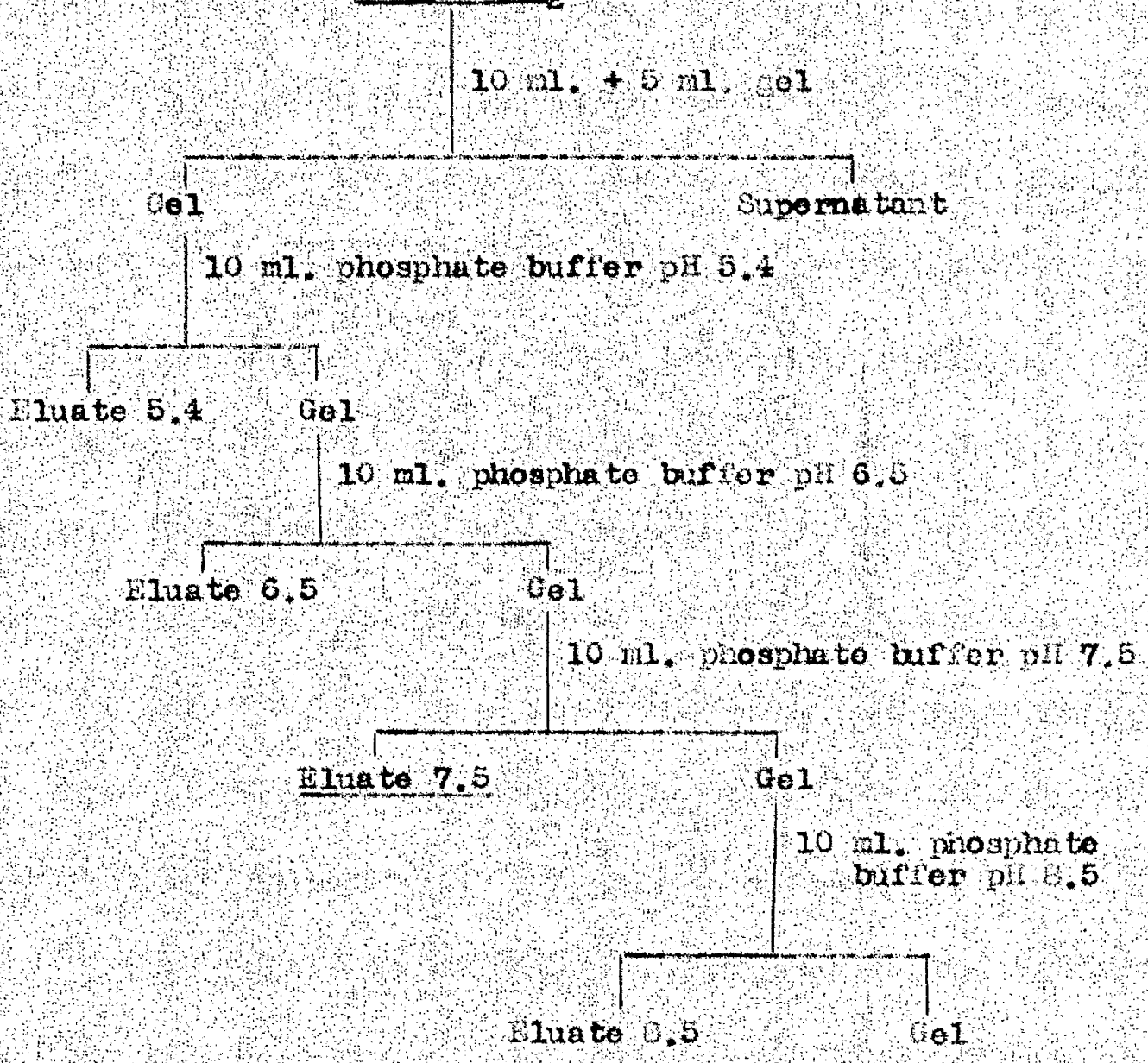

DECREASE IN ABSORPTION AT 340 mμ
.10
.08
.06
.04
.02
0
2
4
6
8
10
12
14
TIME (min.)

If, however, fraction C_2 was precipitated from solution C, then the control activity was considerably reduced. The activity in the extract itself decreased considerably after the extract had been stored for 2 days in the deep freeze, while the alcohol dehydrogenase activity was unaffected. Fig.13 shows the effect of storing protein solution A for 2 days in the deep freeze. The original test showed that protein solution A with acetaldehyde oxidized $DPNH_2$ in 6 min., and that protein solution A alone oxidized the same amount in 11.4 min. After storage, however, when protein solution A had to be cleared by centrifugation the test cuvette gave complete oxidation in 6 min., while the control cuvette, if it continued linearly, would have taken about 24 min., i.e., the activity in the control was reduced 50%. The difference in the possible E_{340} values shown in Fig.13 is due to the fact that the slightest variation in the added volume, and therefore the final concentration of $DPNH_2$, causes a considerable difference in the absorption at 340 mμ.

The effect of pH on the activity of fraction C_2 was investigated. Phosphate buffers of several different pH values were used in the system previously described. The results of four representative pH values are given in Fig.14. At pH 6.5 the activity in the test less the activity in the control gave the highest results. At all

Table 13.

Adsorption and elution of Fraction C_2 from calcium phosphate gel.



Fraction C_2 and the gel were allowed to stand for 20 min. before centrifuging. For the elutions, the buffer and gel were mixed, shaken for 15 min. and then centrifuged.

Fig. 18. The decrease in absorption at 340 mμ due to the oxidation of $TPNH_2$ by acetaldehyde in the presence of the supernatant and eluates from calcium phosphate gel on to which fraction C_2 had been absorbed. The system contained phosphate buffer 6.0 μmoles, $MgSO_4.7H_2O$ 3.12 μmoles, $TPNH_2$ 0.1 μmole, acetaldehyde 10 μmoles and extract 1.0 ml. per 3 ml. ○ eluates pH 5.0, pH 6.5, pH 6.9 and supernatant; ● eluate pH 7.5. Controls, with acetaldehyde omitted, have been subtracted from the figures shown.

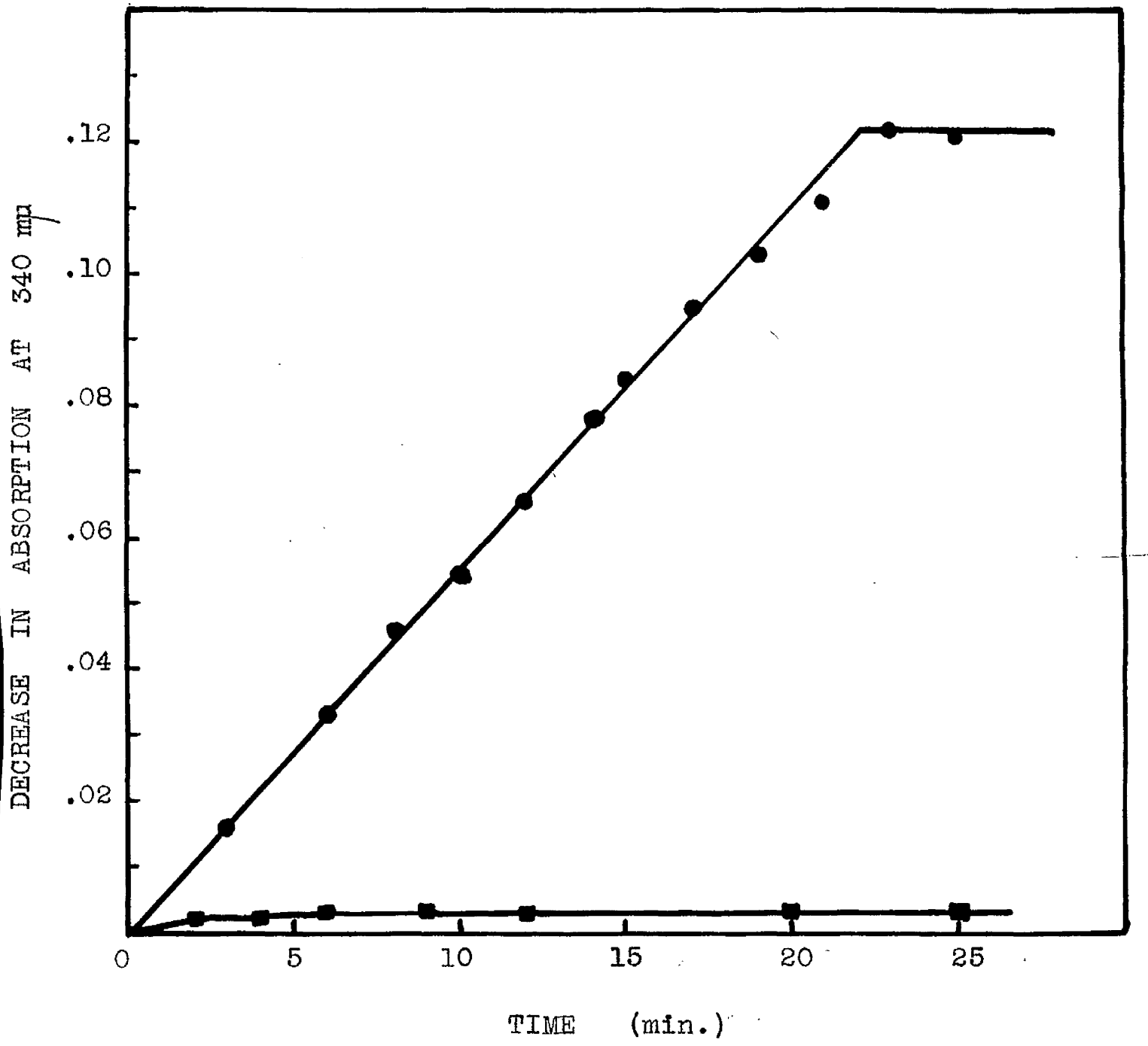
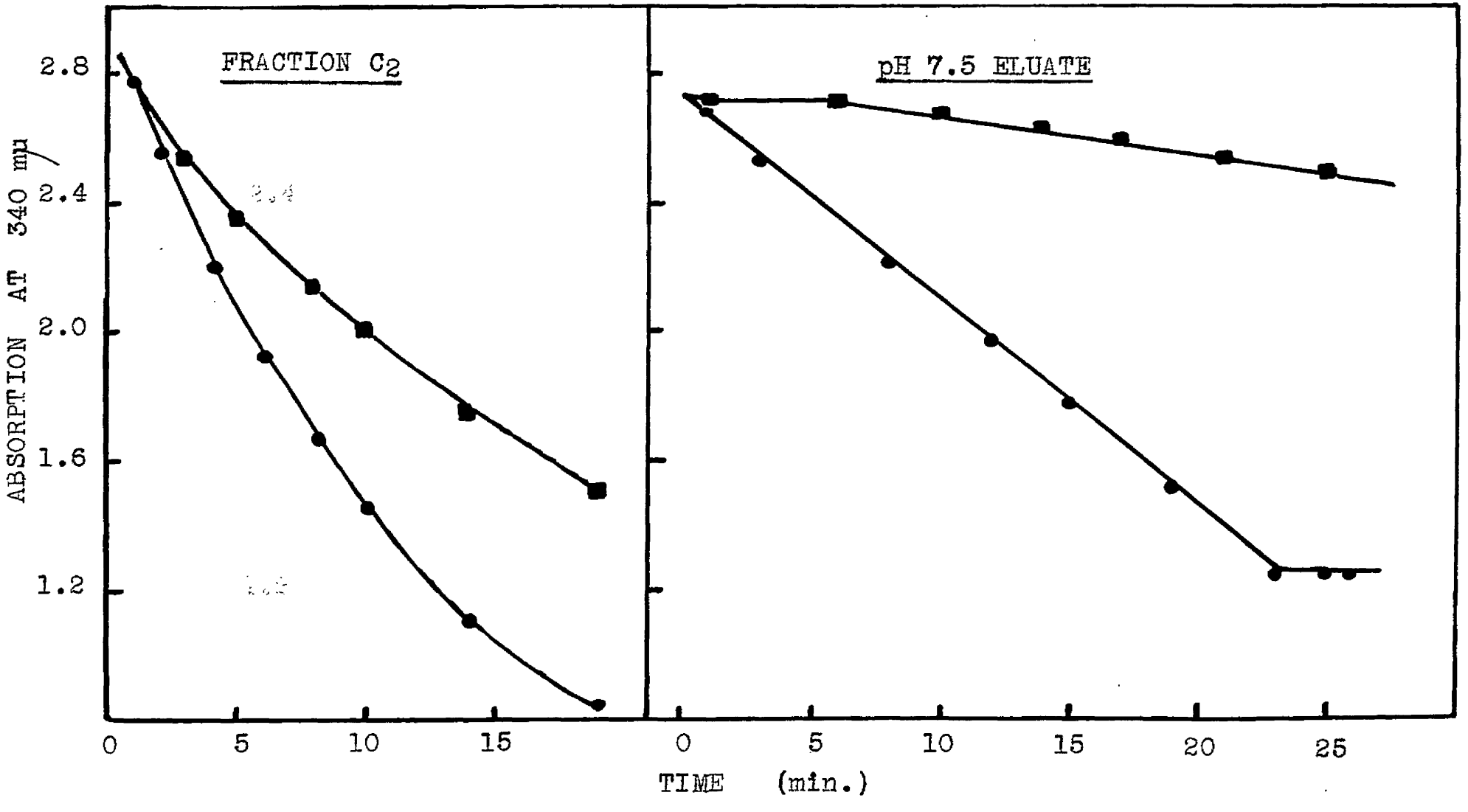

.12
.10
.08
.06
.04
.02
0
5
10
15
20
25
DECREASE IN ABSORPTION AT 340 mμ
TIME (min.)

Fig.10. A comparison of the activity of fraction C_2 and the pH 7.5 eluate in the system phosphate buffer 3.3 µmoles, $MgSO_4.7H_2O$ 3.12 µmoles, $TPNH_2$ 0.1 µmole, acetaldehyde 10 µmoles and extract 1.0 ml. per 3 ml. O Control with acetaldehyde omitted: ● complete system.


FRACTION C_2
pH 7.5 ELUATE
ABSORPTION AT 340 mμ
2.8
2.4
2.0
1.6
1.2
0
5
10
15
0
5
10
15
20
25
TIME (min.)

the other pH values the results were low due to high control readings. 10 ml. of fraction C_2 were treated as shown in Table 10. The fraction was added to 5 ml. calcium phosphate gel, and the mixture allowed to stand 20 minutes. The supernatant, after centrifuging, was decanted and the gel eluted with 10 ml. portions of phosphate buffer, of pH 5.4, 6.5, 7.5 and 8.5 respectively. For elution, the buffer was added to the gel and a smooth suspension made by stirring with a glass rod. This suspension was shaken gently in a 'Microid' shaker for 15 minutes. The eluate was obtained by centrifugation. The original fraction C_2, the supernatant and the eluates were tested in the same system as before, with the pH of the phosphate buffer adjusted so that the final pH of the whole system was 6.5. The activity of these eluates is given in Fig.15. All the activity was adsorbed on to the gel, as shown by the fact that the supernatant from the adsorption was completely inactive. The active fraction was eluted with pH 7.5 buffer and control activity was almost completely lost by this procedure. A comparison of the activity of the original C_2 fraction and the pH 7.5 eluate is given in Fig.16.

This eluate was set up with phosphate buffer, pH 5.0, 6.6 µmoles, acetaldehyde 5 µmoles, $MgSO_4.7H_2O$ 3.12 µmoles, $DPNH_2$ 0.2 µmoles and extract 1.0 ml. per 3 ml.

1.17. The reversible reduction of acetaldehyde to ethanol in the presence of the pH 7.5 eluate and $DPNH_2$. The system contained phosphate buffer pH 6.0 3.3 μmoles, acetaldehyde 5 μmoles, $MgSO_4.7H_2O$ 3.12 μmoles, $DPNH_2$ 0.2 μmole, and eluate 1 ml. per 3 ml. At 15 min. 30 μmoles ethanol were added to the ■ test cuvette: an equal volume of water was added to the ● control cuvette.

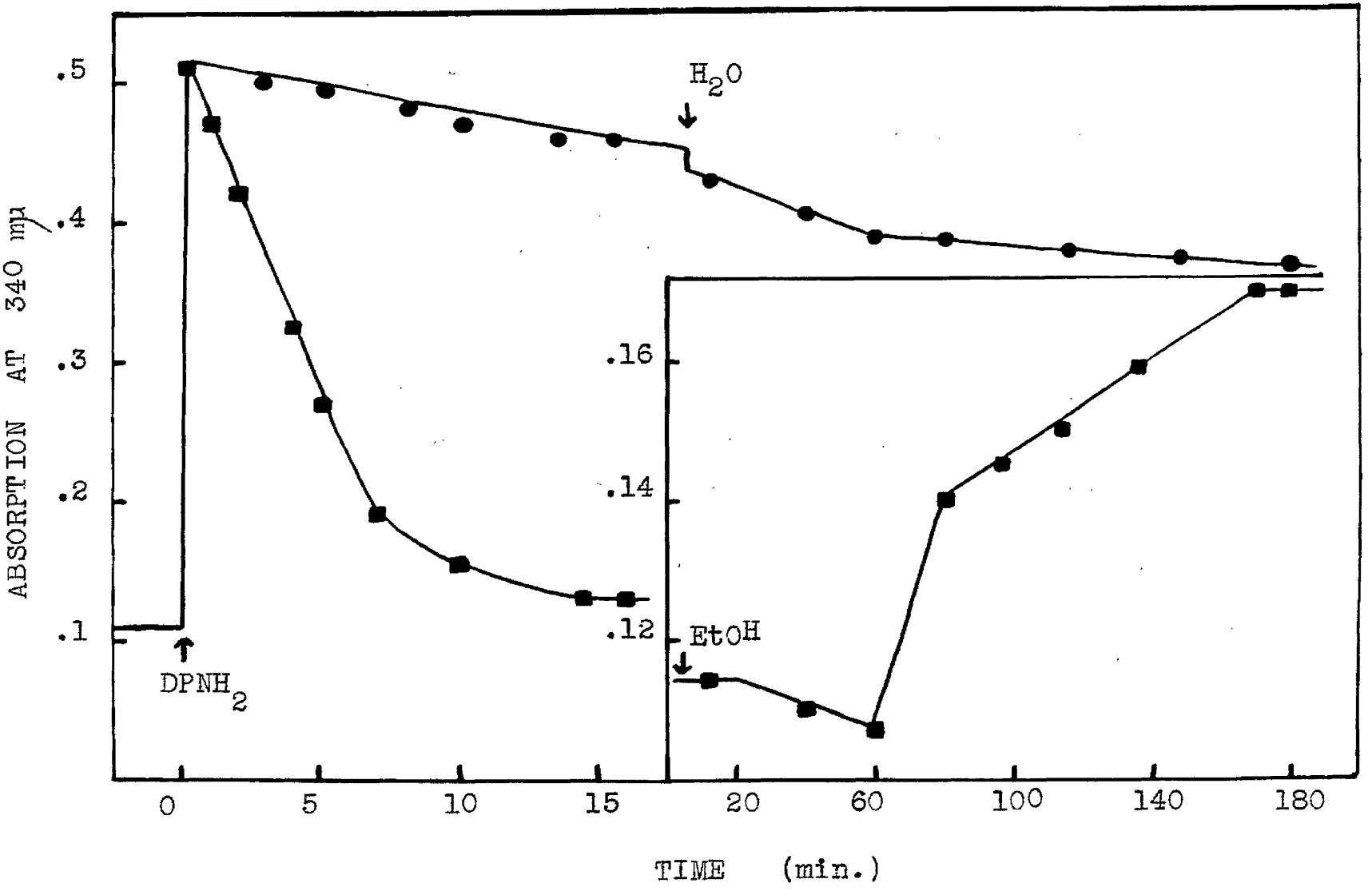

.5
.4
.3
.2
.1
ABSORPTION AT 340 mμ
H_2O
$DPNH_2$
.16
.14
.12
EtOH
0
5
10
15
20
60
100
140
180
TIME (min.)

Table 19.

Oxidation of acetaldehyde by an extract obtained after grinding freeze-dried powder with alumina. Contents of the Warburg flasks were as follows:

	Autorespiration	Acetaldehyde Control	Acetaldehyde	(3) + CoA + DPN	(1) + CoA + DPN	Thermobarometer
	(1)	(2)	(3)	(4)	(5)	(6)
Phosphate buffer	0.6	0.6	0.6	0.6	0.6	-
Acetaldehyde	-	1.0	1.0	1.0	-	-
Extract	0.6	-	0.6	0.6	0.6	-
DPN	-	-	-	0.2	0.2	-
Water	0.2	1.0	0.2	-	-	3.0
KOH	0.2	0.2	0.2	0.2	0.2	-
CoA (in buffer)	-	-	-	0.2	0.2	-

Fig. 1. Oxidation of acetaldehyde by an extract obtained after grinding freeze-dried cells of Escherichia coli with alumina. The contents of the Warburg flasks are given in Table 10. The final concentrations were acetaldehyde 100 μmoles, DPN 0.2 μmole and CoA 30 units. ○ Autorespiration, ● acetaldehyde + CoA + DPN, ■ all other flasks.

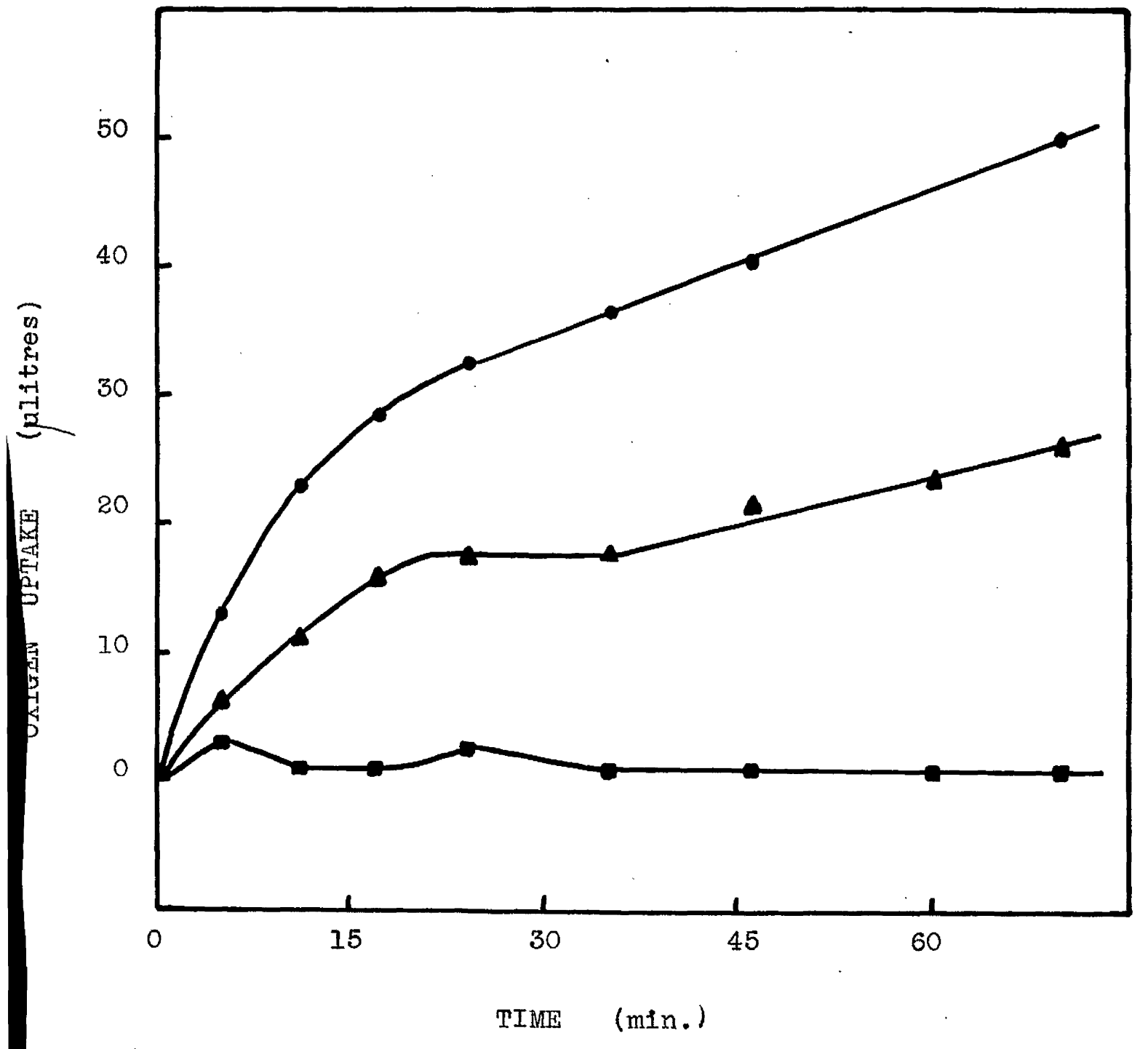
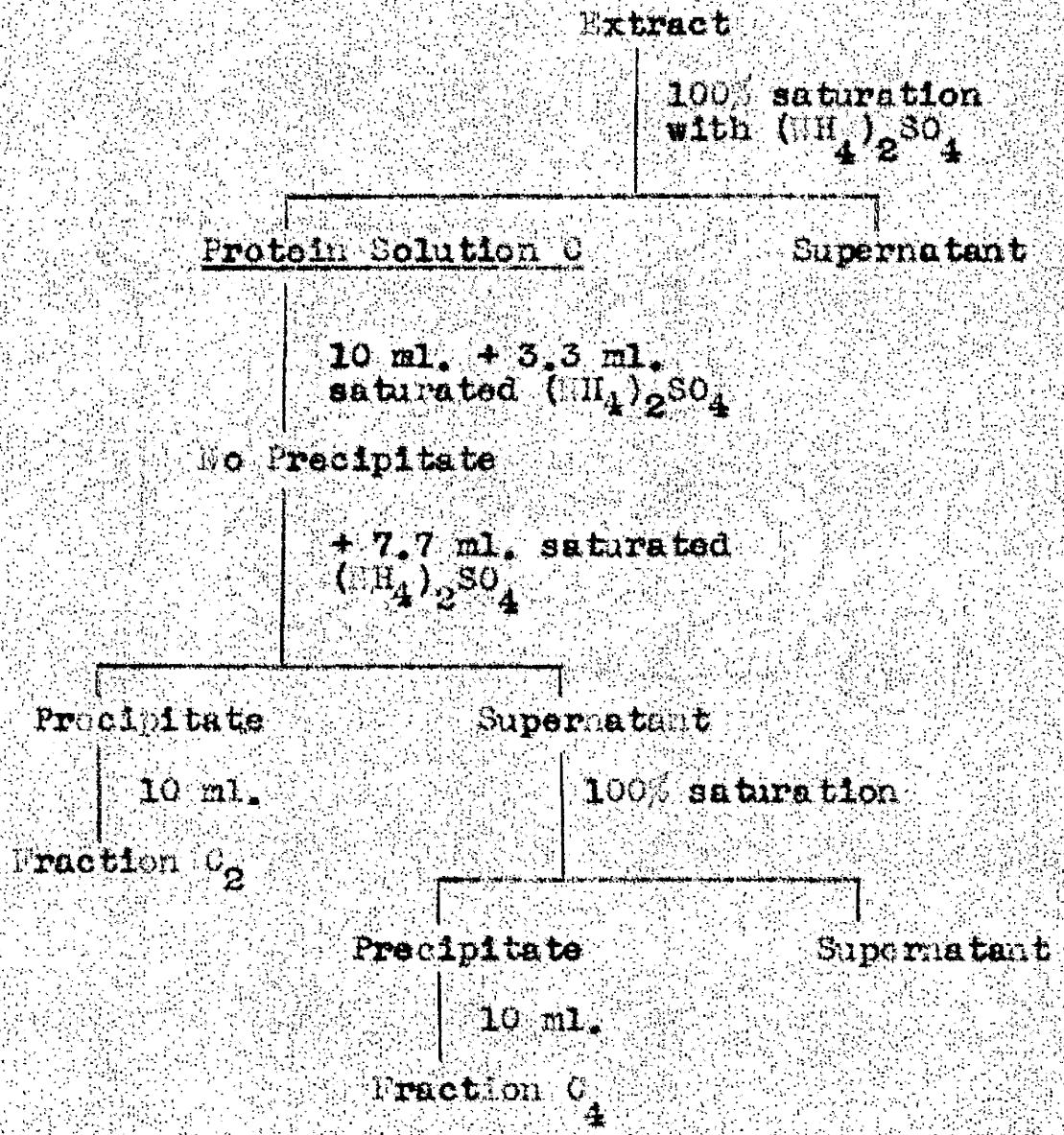

50
40
30
20
10
0
(µlitres)
0
15
30
45
60
TIME (min.)

Table 20.

Fractionation of Protein Solution C for experiments on the reduction of acetyl phosphate or acetic acid.



The $DPNH_2$ was reduced in 12.5 minutes. The initial reading of the system minus $DPNH_2$ was 0.11: after 12.5 minutes the reading had decreased from 0.525 at zero time to 0.135. At 15 minutes ethanol, 30 µmoles, was added to the test cuvette and an equal volume of water to the control cuvette. At 60 minutes the absorption at 340 mµ began to increase showing the $DPNH_2$ was being reformed. The reverse reaction, which had a very much slower rate than the forward reaction, continued for 2 hours. This is shown in Fig.17.

Oxidation of acetaldehyde.

1 g. of freeze dried powder was ground with 2 g. alumina and extracted with 15 ml. chilled phosphate buffer. The extract was set up in Warburg manometric flasks as shown in Table 19. The final concentrations in the system were: acetaldehyde 100 µmoles, DPN 0.2 µmoles and CoA 30 units. Fig.18 shows that in the presence of DPN and CoA there was slight oxidation of acetaldehyde.

The reduction of acetic acid and acetyl phosphate.

5 g. wet weight cells were crushed and extracted with 30 ml. phosphate buffer. This was fractionated as shown in Table 20. The fractions so obtained were tested in the system phosphate buffer 6.6 µmoles, $MgSO_4.7H_2O$ 3.12 µmoles, CoA 30 units, glutathione 5 µmoles, ATP

0.21 µmoles, $DPNH_2$ 0.1 µmole and acetyl phosphate 5 µmoles or sodium acetate 5 µmoles per 3 ml. A control was set up with no substrate. The reduction of $DPNH_2$ by the total protein and by fraction C_2 was the same in the control cuvette as in the cuvettes containing acetyl phosphate and sodium acetate. Wood and Schwert (1954) found that $DPNH_2$ was oxidized by the dissolved oxygen in their cuvettes in the presence of an extract of Pseudomonas fluorescens and excess glucose-6-phosphate, which was reducing DPN to $DPNH_2$. Only when the dissolved oxygen was fully utilized did $DPNH_2$ start to accumulate. This could be reversed by aerating the cuvettes with a stream of air bubbles. The oxidation in the control cuvette in this experiment might have been due to dissolved oxygen. An attempt to inhibit this oxidation by gently bubbling nitrogen through the solution in the cuvette met with no success. Some of the fractions and eluates used for the reduction of acetaldehyde were tested to see if they would reduce acetate or acetyl phosphate. It was found, however, that as the control activity was removed, so the activity in the presence of acetate or acetyl phosphate was reduced. Thus the fractions were completely inactive with these two substrates.

DISCUSSION.

There are many variable factors which influence the end products arising from glucose metabolism. The conditions under which the cells are grown have been found to affect the amount of ethanol produced from glucose by washed cell suspensions. Cells grown in glucose-ammonium salt medium produced only half the amount of ethanol that was produced by cells grown in the medium to which traces of peptone and yeast extract have been added. Some uncontrollable factor must also affect the production of ethanol. 'Inactive' cells were obtained which produced only 25% of the ethanol produced by active cells from the same amount of glucose under apparently identical conditions.

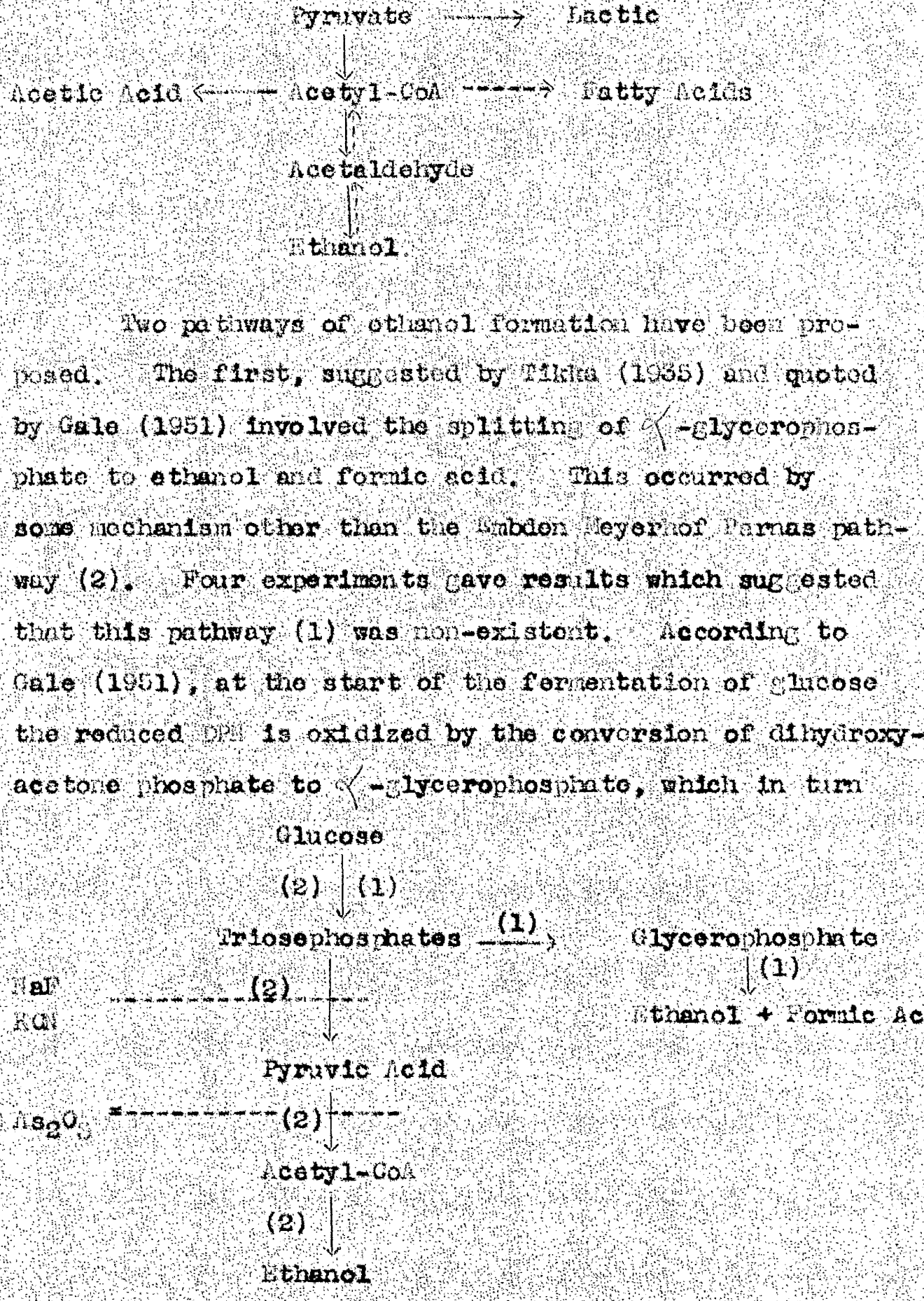
It would seem that glucose fermentation cannot be expressed by definite equations. Three equations have been drawn up for the fermentation of glucose by Esch. coli. These have been obtained under different conditions and are in each case different. Harden (1901) worked with growing cultures, although his findings were later confirmed with cell suspensions by, for example, Grey (1913, 1914, 1919). These early analyses were carried out with more empirical methods than are used today, which might possibly account for the differences in some of the results, although the discrepancy in the lactic acid concentrations seems rather too large to be dismissed as

	Harden	Stokes	Foster
Glucose	1	1	1
Acid	-	2.5	2.50
Lactic Acid	1.0	0.2	0.02
Acetic Acid	0.5	0.8	0.69
Ethanol	0.5	0.8	0.74
Succinic Acid	Trace	0.4	-
Formic Acid	-	1.2	0.03
Acetic:Ethanol	1:1	1:1	1:1.07
Ethanol:Glucose	0.5:1	0.8:1	0.74:1

experimental error. Stokes (1949) worked with cell suspensions of Esch. coli, whereas the equation drawn up in this present work is based on the results of an analysis of the supernatant of a culture of Esch. coli grown in glucose-ammonium salt medium with a limiting concentration of glucose. The total acid produced, the ethanol and acetic acid figures agree well with those of Stokes but the lactic acid and formic acid figures are very different. There is a discrepancy of 1.02 moles between the total acid produced in our cultures per mole of glucose and the sum of those estimated. Assuming that 2.5 moles acid per mole of glucose were produced in Harden's fermentations, the sum of the estimated acids in this case is also 1 mole less than the total. Harden (1901) found only traces of

succinic acid, whereas Stokes (1949) found 0.4 moles succinic acid. If the same amount of succinic acid was present in the supernatant of the present experiment, there is still 0.6 moles of acid to be accounted for. When cell suspensions were used, the ethanol:glucose ratio was usually in good agreement with 0.74:1. However, the acetic acid:ethanol ratio was not 1:1. In fact although the ethanol rose to 10 µmoles/ml. the acetic acid concentration remained below 4 µmoles/ml.

Harden (1901) postulated that the acetic acid and ethanol arose from a common precursor. This is now known to be acetyl-CoA. The ethanol concentration always reached a maximum and then declined. It seemed probable that when the ethanol concentration decreased, acetyl-CoA would be reformed and that this might yield acetic acid, giving a rise in acetic acid concentration corresponding to the decrease in the ethanol concentration. This was not established. Neither could an increase in any of the other tested end products be detected. Stadtman and Barker (1949) showed that Cl. kluyveri utilized ethanol, firstly giving acetaldehyde, and acetic acid via acetyl-CoA to synthesize fatty acids. Whether this occurs in Esch. coli cultures is not known.



Two pathways of ethanol formation have been proposed. The first, suggested by Tikka (1935) and quoted by Gale (1951) involved the splitting of α-glycerophosphate to ethanol and formic acid. This occurred by some mechanism other than the Embden Meyerhof Parnas pathway (2). Four experiments gave results which suggested that this pathway (1) was non-existent. According to Gale (1951), at the start of the fermentation of glucose the reduced DPN is oxidized by the conversion of dihydroxyacetone phosphate to α-glycerophosphate, which in turn

yields ethanol and formic acid. As the fermentation proceeds pyruvic acid is produced and this replaces the triose phosphate as a hydrogen acceptor. Thus with the production of pyruvic acid, the production of α-glycerophosphate, and so of ethanol, should cease. The amount of ethanol produced will, therefore, depend on the speed of pyruvate production. When a washed cell suspension of Esch. coli is added to phosphate buffer and glucose there is almost instantaneous production of pyruvic acid. The production of ethanol, on the other hand, is often difficult to detect until about 20 to 30 minutes after the cell suspension has been added.

If the Embden Meyerhof Parnas pathway for glucose fermentation is blocked so that triosephosphate cannot be converted to pyruvate then, if this other pathway does exist, one would expect the triosephosphate to be shunted through α-glycerophosphate to ethanol and formate. When NaF and KCN were added to inhibit the production of pyruvate, and As_2O_3 to inhibit the utilization of pyruvate, then ethanol production was also inhibited. An extract was prepared from crushed cells which fermented glucose and glycerol to pyruvic acid, which accumulated. The extract would not dissimilate pyruvic acid. In the last experiment, an extract, prepared from acetone dried powder

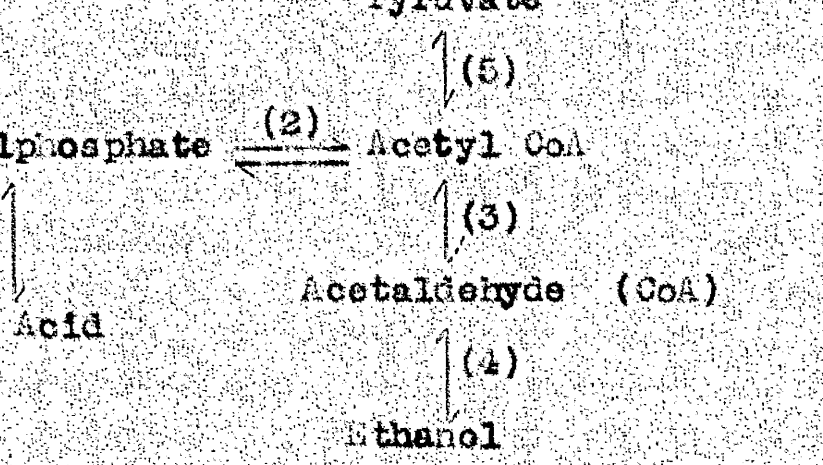
and which fermented α-glycerophosphate to ethanol, was shown to be CoA dependent. The conclusion reached from these three experiments is that either the α-glycerophosphate pathway is the Embden Meyerhof Parnas one, or it is so similar that it is inhibited by the same inhibitors, leads through pyruvic acid and requires CoA.

Glucose	Glycerophosphate
↓	↓
Pyruvate	Pyruvate
↓	↓
Acetyl CoA	Acetyl CoA
↓	↓
Ethanol	Ethanol

Many workers, as previously reported, have now shown that glycerol and α-glycerophosphate are metabolized <u>via</u> dihydroxyacetone phosphate to pyruvic acid. There is still some doubt as to whether glycerol proceeds by a non-phosphorylated pathway to pyruvic acid in some bacteria, or whether the first step is always phosphorylation, either of glycerol to α-glycerophosphate or of dihydroxyacetone to dihydroxyacetone phosphate. The important point is, however, that pyruvic acid is produced either by the Embden Meyerhof Parnas pathway, or one extremely similar, and that the ethanol is produced from this.

The second pathway proposed for ethanol formation

arises from pyruvic acid and proceeds through acetyl-CoA and acetaldehyde or its CoA complex to ethanol. The importance of pyruvic acid in the formation of ethanol was shown by the use of inhibitors. Inhibitors which blocked the production or the utilization of pyruvate also blocked the formation of ethanol. Experiments with a pantothenate-requiring mutant of Esch. coli showed that ethanol formation depended on the dissimilation of pyruvate and on CoA. The slower the rate of pyruvate dissimilation the slower the rate of ethanol formation. The rate of pyruvate dissimilation was determined by the CoA content of the cells.



The enzymes required to produce ethanol from pyruvate by this proposed pathway are the phosphoroclastic splitting enzyme (5), acetaldehyde dehydrogenase (3) and alcohol dehydrogenase (4) as shown in the scheme. It

was very easy to obtain an extract from Esch. coli which contained an active alcohol dehydrogenase (4). Still (1940) found that his preparation of alcohol dehydrogenase had an optimum pH 7.4 for the oxidation of alcohol. When the reduction of acetaldehyde was followed, pH 6.5 was found to give the highest activity in this work. The active fraction was eluted from calcium phosphate gel with phosphate buffer pH 7.5. There was a considerable difference between the speed of acetaldehyde reduction and ethanol oxidation. The reaction could only be reversed if a large excess of ethanol was added, 50 µmoles ethanol to a system containing 5 µmoles of acetaldehyde. This indicates that the equilibrium of the reaction must be very much towards the formation of ethanol which could possibly explain the difficulty encountered in the detection of free acetaldehyde in fermentations without the use of fixative compounds.

These same extracts which contained enzyme (4) showed a very slight activity for the oxidation of acetaldehyde. This oxidation depended on the presence of CoA and DPN, indicating that acetyl-CoA was being formed under the action of acetaldehyde dehydrogenase (5). Burton and Stadtman (1953) have studied the acetaldehyde dehydrogenase of Ol. kluyveri. Pinchot and Racker (1951) have already shown that Esch. coli extracts could oxidize

ERRATUM : p. 87, lines 10, 11.
For 'alcohol' read 'acetaldehyde'.

acetaldehyde in the presence of CoA, but they did not demonstrate the reverse reaction. The extracts used to study alcohol dehydrogenase were tested for the reduction of acetic acid through (1), (2), (3) and (4), and of acetyl phosphate through (2), (3) and (4). There was no activity. Thus, either phosphotransacetylase (2) or acetaldehyde dehydrogenase (3) was missing or inactive. Such extracts dissimilated pyruvate to acetic and formic acid, with no ethanol formation. Thus reactions (3), (2) and (1) must have functioned, and this indicates that alcohol dehydrogenase (5) is the enzyme which is preventing the reduction of acetylphosphate to ethanol. 'Inactive' cells of Esch. coli, which fermented glucose at the same rate as active cells but which produced no ethanol, were unable to oxidize ethanol either in an atmosphere of H_2 or air. This would indicate that such cells were deficient in enzymes (3) or (4). In view of the work with cell-free extracts, it seems most probable that the inactive enzyme is acetaldehyde dehydrogenase.

It was intended to carry out some isotopic experiments with labelled pyruvic acid which, had they yielded the desired result, would have shown that ethanol arises from carbon atoms 2 and 3 of pyruvic acid. The main reason why such experiments were never performed was the

baffling and too frequent occurrence of an inactive batch of cells. Other reasons, for example, extracts which did not produce ethanol from glucose and the non-production of ethanol from the dissimilation of pyruvic acid, thwarted this intention.

The presence of an active alcohol dehydrogenase and of an acetaldehyde dehydrogenase has been shown in Esch. coli extracts. Ample evidence exists of the production of acetyl-CoA from pyruvate in Esch. coli. Thus the three enzymes are known to exist in the organism. The starting point of the scheme, pyruvic acid, has been shown to lie on the pathway of ethanol production and CoA has been shown to be essential for the production of ethanol. It is very tempting to collate these pieces of evidence and to declare a proven theory. In fact, these results merely lend support to the proposed pathway and more overall evidence is required before the scheme is firmly established. This could be furnished by the use of isotopes but, until such experiments are carried out, the proposed pathway must remain a working hypothesis.

This work was undertaken to establish the mechanism of ethanol production. It has provided several pieces of positive evidence in favour of the proposed scheme. It has also produced many negative results, but at no time

has any evidence accrued to indicate that the suggested pathway is wrong. Thus, in the absence of any truly negative evidence and in the presence of some very definitely positive indications, it may be suggested that the proposed pathway will, in all probability, eventually be established as the route for ethanol formation in *Esch. coli*.

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